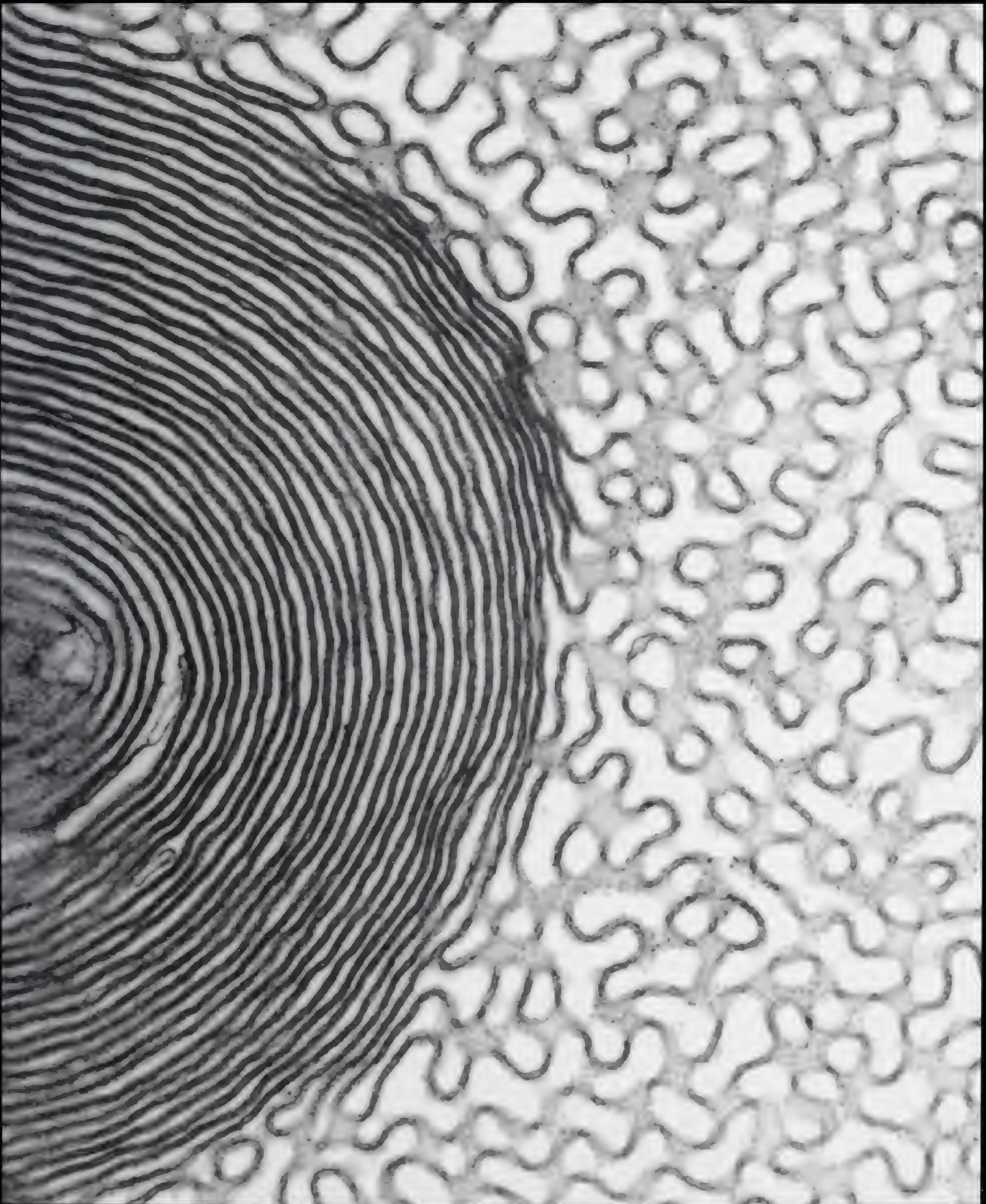
2003 Annual Report of the Division of Intramural Research



National Institute of Child Health and Human Development

COVER

Though normally seen as a branching network of membranes, the endoplasmic reticulum (ER) can quickly and dramatically reorganize into a variety of regular, tightly stacked arrays in response to elevated levels of specific resident proteins, such as cytochrome b(5). Overexpression of these proteins induces the formation of karmellae, whorls, and crystalloids, collectively referred to as organized smooth endoplasmic reticulum (OSER). This image is a transmission electron micrograph of a CV-1 cell overexpressing cytochrome b(5), and is an example of a whorl surrounded by radiating sinusoidal ER. Use of chimeric resident ER membrane proteins tagged with green fluorescent protein (GFP) and photobleaching revealed that OSER can form as a consequence of transient low-affinity protein interactions. In fact, the addition of GFP, which undergoes low-affinity, antiparallel dimerization, to the cytoplasmic domains of non-OSER–inducing resident ER proteins was sufficient to induce OSER structures when overexpressed, but the addition of a nondimerizing GFP variant was not. The mass action of weakly binding proteins might be a general way to organize stacked organelle structures, including chloroplast thylakoids, the myelin sheath, and the Golgi apparatus. The normal function of OSER is still unknown, but the structures may help sequester lipophilic drugs away from other organelles or regions of the cytoplasm during detoxification. In addition, the observation that OSER whorl structures can form when mutant membrane proteins accumulate in the ER as a consequence of defects in their ability to be exported out of the ER, as is the case in the pathogenic phenotype observed in a mouse model of Charcot-Marie-Tooth syndrome as well as in early-onset torsion dystonia, suggests the involvement of OSER in pathogenesis (also see page 22).

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March 9, 2004

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Phil

Dear Dr. Chen:

Attached is the NICHD 2003 Annual Report of the Division of Intramural Research for your interest.

Sincerely,

Owen

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NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

DIVISION OF INTRAMURAL RESEARCH

ANNUAL REPORT

2003

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health

CONTENTS

BOARD OF SCIENTIFIC COUNSELORS vi

PREFACE v

INTRODUCTION vii

RESEARCH PROJECTS OF THE LABORATORIES AND BRANCHES 1

1. BONE AND EXTRACELLULAR MATRIX BRANCH

Connective Tissue Disorders *Joan Marini* 3

2. CELL BIOLOGY AND METABOLISM BRANCH

Protein Sorting in the Endosomal-Lysosomal System *Juan Bonifacino* 6
Regulation of Secretory and Membrane Protein Biogenesis *Ramanujan Hegde* 10
Regulation of Membrane Dynamics and Protein Transport by the Sec7-Domain Guanine Nucleotide Exchange Factors *Catherine Jackson* 14
Cell Cycle Regulation During Oogenesis *Mary Lilly* 17
Dynamics of Secretory Membrane Trafficking, Sorting, and Compartmentalization *Jennifer Lippincott-Schwartz* 21
Regulation of Intracellular Iron Metabolism *Tracey Rouault* 25
Microbial Responses to Oxidative Stress and Noncoding RNAs *Gisela Storz* 28

3. DEVELOPMENTAL ENDOCRINOLOGY BRANCH

Neuroendocrinology of Stress *Greti Aguilera* 31
Regulation of Skeletal Growth *Jeffrey Baron* 35
Genes or Hormones? Gender-Specific Differences in Disease Susceptibility *Carolyn Bondy* 39
The Menstrual Cycle and Human Reproduction *Lawrence Nelson* 44
Molecular Genetics of Adrenocortical Tumors and Related Disorders *Constantine Stratakis* 48
Physiology, Psychology, and Genetics of Obesity *Jack Yanovski* 54

4. ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH

Phosphoinositide-Calcium Signaling in Cell Regulation *Tamas Balla* 58
Peptide Hormone Receptors and Signal Transduction *Kevin Catt* 61
Structure and Function of Peptide and Protein Hormones *Hao-Chia Chen* 66
Gonadal Receptors and Mechanism of Action of Peptide Hormones in Steroidogenic Cells *Maria Dufau* 68
Signal Transduction in Synaptic Transmission and Plasticity *Kuo-Ping Huang* 72
Calcium Signalling and Calcium-Controlled Cellular Functions *Stanko Stojilkovic* 76
The Biotransformation of Endobiotics by Sulfonation *Charles Strott* 81

5. HERITABLE DISORDERS BRANCH

Molecular Genetics of Heritable Human Disorders *Janice Chou* 86
Heritable Autoimmune and Neurodegenerative Disorders *Anil Mukherjee* 89
Molecular Biology, Regulation, and Biochemistry of UDP-Glucuronosyltransferase Isozymes *Ida Owens* 94
Dysmorphic Syndromes and Birth Defects *Forbes Porter* 97

6. LABORATORY OF CELLULAR AND MOLECULAR BIOPHYSICS

Protein-Mediated Remodeling of Membrane Lipid Bilayers *Leonid Chernomordik* 101
Advances in the Methodology of Electrophoretic Separation *Andreas Chrambach* 104
Microbial Pathogens in Human Lymphoid Tissue *Leonid Margolis* 107
Applications of Mass Spectrometry to Biophysics, Biochemistry, and Medicine *Alfred Yergey* 111
Membrane Remodeling During Pathogenesis and the Mechanism of Exocytosis *Joshua Zimmerberg* 115

7. LABORATORY OF CELLULAR AND MOLECULAR NEUROPHYSIOLOGY

Structure and Function of Glutamate Receptors *Mark Mayer* 119

8. LABORATORY OF CELLULAR AND SYNAPTIC NEUROPHYSIOLOGY

Potassium Channels and Dendritic Function in Hippocampal Pyramidal Neurons *Dax Hoffman* 122
Neurotrophic Regulation of Synapse Development and Plasticity *Bai Lu* 124
Hippocampal Interneurons and their Role in Controlling Excitability *Chris McBain* 128
Olfactory Coding and Decoding by Ensembles of Neurons *Mark Stopfer* 132

9. LABORATORY OF CLINICAL GENOMICS

Clinical Genomics *Owen Rennert* 133
Bridging the Gap between Monogenic Traits and Complex Inherited Disorders of Childhood *Stephen Kaler* 139

10. LABORATORY OF COMPARATIVE ETHOLOGY

Child and Family Development in the First Two Decades of Life *Marc Bornstein* 142
Child Development in Context *Michael Lamb* 147
Mechanisms Regulating Auditory Communication in Primates *John Newman* 151
Genetic and Environmental Determinants of Biobehavioural Development *Stephen Suomi* 154

11. LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY

Vaccines Against Human Pathogenic Bacteria *John Robbins* 158
Human Immune Response to Polysaccharide-Protein Conjugate Vaccines *Rachel Schneerson* 160

12. LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

Developmental Regulation of Neuronal and Muscle Plasticity *Andres Buonanno* 165
Regulation of Pineal Function *David Klein* 168
Secretory Protein Trafficking and Granule Biogenesis in Neuroendocrine Cells *Peng Loh* 174
Tetanus and Botulinum Neurotoxins and Neuronal Cell Biology *Elaine Neale* 178
Mechanisms of Neurodevelopment in Health and Disease *Phillip Nelson* 180

13. LABORATORY OF GENE REGULATION AND DEVELOPMENT

Mitotic Regulation in Higher Eukaryotes by Ran and SUMO-1 *Mary Dasso* 183
Mechanism and Regulation of Eukaryotic Protein Synthesis *Thomas Dever* 186
Transcriptional and Translational Control Mechanisms in Nutrient Regulation of Gene Expression *Alan Hinnebusch* 190
Epigenetic Gene Silencing *Rohinton Kamakaka* 196
Molecular Mechanism of Neuronal Connectivity *Chi-Hon Lee* 198
Retrotransposons as Models for the Replication of Retroviruses *Henry Levin* 202
Molecular Mechanism of Frog Metamorphosis *Yun-Bo Shi* 206
Control of Chromosome Transmission Fidelity by the SMC Protein Complexes *Alexander Strunnikov* 209

14. LABORATORY OF GENOMIC INTEGRITY

Studies on DNA Replication, Repair, and Mutagenesis in Eukaryotic and Prokaryotic Cells *Roger Woodgate* 211

15. LABORATORY OF INTEGRATIVE AND MEDICAL BIOPHYSICS

Tissue Biophysics and Biomimetics *Peter Basser* 217
Medical Biophysics *Robert Bonner* 224
Applications of Photon Migration to Tissue Tomography and Spectroscopy *Amir Gandjbakhche* 228
Cell Biophysics *Ralph Nossal* 234

16. LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT

Signals Regulating T Cell Development *Paul Love* 237
Molecular Genetics of an Imprinted Gene Cluster on Mouse Distal Chromosome 7 *Karl Pfeifer* 241
Mammalian Developmental Genetics *Heiner Westphal* 244

17. LABORATORY OF MOLECULAR GENETICS

Nutrient Control of Bacterial Gene Expression *Michael Cashel* 247
Patterning Neurogenesis in the Zebrafish Embryo *Ajay Chitnis* 250
Physiological, Biochemical, and Molecular Genetic Events of Recognition and Resolution of RNA-DNA Hybrids *Robert Crouch* 254
Molecular Genetics of Embryogenesis in *Xenopus* and Zebrafish *Igor Dawid* 257
Control of Gene Expression During Development *Judith Kassis* 260
Regulation of Homeotic Genes in *Drosophila, James Kennison* 263
Molecular Genetics of Mammlian Retrovirus Replication *Judith Levin* 266
Regulation of Ectodermal Development in *Xenopus, Thomas Sargent* 270
Organogenesis of the Zebrafish Vasculature *Brant Weinstein* 273
Mechanisms for Controlling Gene Expression in Bacteria and Bacteriophage *Robert Weisberg* 278

18. LABORATORY OF MOLECULAR GROWTH REGULATION

Gene Activation in Yeast: Targeted Histone Acetylation and Remodeled Chromatin Domains *David Clark* 282
Regulation of DNA Replication and Gene Expression During Animal Development *Melvin DePamphilis* 286
Mapping Higher Order Chromatin Structures *Bruce Howard* 290
Mechanisms of Eukaryotic RNA Expression and Functions of the Human La Antigen *Richard Maraia* 293
Developmental Gene Regulation of the Immune System *Keiko Ozato* 296

19. LABORATORY OF PHYSICAL AND STRUCTURAL BIOLOGY

Biophysics of Large Membrane Channels *Sergey Bezrukov* 300
Physical Forces Organizing Biomolecules *Adrian Parsegian* 303
Intermolecular Forces, Recognition, and Dynamics *Donald Rau* 306

20. PEDIATRIC AND REPRODUCTIVE ENDOCRINOLOGY BRANCH

The Hypothalamic-Pituitary-Adrenal Axis: its Endocrine Disorders and Interactions with the Immune System *George Chrousos* 309
Hypercortisolism and Reproductive Disorders *Lynnette Nieman* 316
Diagnosis, Localization, Pathophysiology, and Molecular Biology of Pheochromocytoma *Karel Pacak* 320
Reproductive Endocrinology and Infertility *James Segars* 325

21. PERINATOLOGY RESEARCH BRANCH

Pathophysiology of Premature Labor, Complications of Prematurity, and Prenatal Diagnosis of Congenital Anomalies *Roberto Romero* 327

22. PHYSICAL DISABILITIES BRANCH

Research in Physical Disabilities *Steven Stanhope* 332

23. SECTION ON PHYSICAL BIOCHEMISTRY

Physical Principles of Biomolecular Recognition, Self-Assembly, and Regulation *Sergey Leikin* 342

24. SECTION ON NERVOUS SYSTEM DEVELOPMENT AND PLASTICITY

Nervous System Development and Plasticity *Douglas Fields* 346

25. SECTION ON CELL BIOLOGY AND SIGNAL TRANSDUCTION

Calcium-Based Excitability in Glial Cells *James Russell* 350

INDEX OF PRINCIPAL INVESTIGATORS 353

BOARD OF SCIENTIFIC COUNSELORS

William W. Chin, MD, Chair	Vice President, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN
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William B. Rizzo, MD	Director, Hobart Wiltse Center for the Study of Metabolic Diseases, Children's Hospital, and Head, Section of Inherited Metabolic Diseases, Department of Pediatrics, Nebraska Medical Center, Omaha, NE
Stephen J. Smith, PhD	Professor, Department of Molecular and Cellular Physiology, Stanford School of Medicine, Stanford, CA
Kenneth S. Zaret, PhD	Senior Member, Basic Science Division, Cell and Developmental Biology Program, Fox Chase Cancer Center, Philadelphia, PA

SCIENTIFIC DIRECTOR'S PREFACE

"The essential mystery remained. How could a monotonous substance such as DNA, like an alphabet with only four letters, convey enough specific information to produce the enormous variety of living things, from daisies to dinosaurs? The answer must be in the way the molecule was put together." Brenda Maddox citing Erwin Schrödinger in "Rosalind Franklin, The Dark Lady of DNA," 2002

"To understand the molecular mechanisms that allow life to begin is to understand more clearly what we are, where we have been, and where we are going." Cited in "Rosalind Franklin, The Dark Lady of DNA."

The Division of Intramural Research of the National Institute of Child Health and Human Development has as its focus the study of the biology of development. It poses several fundamental questions:

- How does the cell communicate/signal from its exterior margins (cell membrane) to its cytosolic compartment (an aqueous environment filled with various organelles and macromolecules) and then to the nucleus to initiate gene expression and replication? Subsequently, how does it translate these chemical and molecular network responses to produce changes in function, differentiation and, in turn, signal to its nearest neighbors and the environment?

- How do cells talk to one another? How do they identify their location and properties to one another and thus give rise to tissues, organs, and an organism?

- How are these processes integrated during embryonic, fetal, and postnatal development? When these processes go awry, how does disease arise, and how may we intervene in the sequence to prevent, diagnose, and treat diseases of children and women?

"The phenomenon of embryologic development and differentiation is generally regarded as one of the two most profound unsolved problems in human biology—the other being the operation of the brain. In both cases, the core of the mystery is the cooperative and collaborative behavior of the cells themselves. The embryo develops from a single cell into an elaborately complex structure, a baby, made up of trillions of cells, each one specialized for doing what it is supposed to do and confining its activities to its designated area but kept in communication with all the rest by chemical advertisements." Lewis Thomas "The Fragile Species," 1992

Science is the pursuit of information about the natural world to enrich man's capacity to understand the universe we exist in. Science cannot be divided into basic and clinical research—there is only research that has or has not yet been applied. Biomedical environments where science flourishes and is the centerpiece ensure that the medicine of today will be replaced by the medicine of tomorrow. Only when medicine is recognized to be the practice of humane scholarship, the discipline devoted to understand, treat and cure disease for all humanity, will the impact of research be translated into *true* clinical practice.

The outstanding scientists of the Division of Intramural Research of NICHD have committed themselves to the pursuit of these objectives. They have made milestone contributions in areas such as vaccine development (*H. influenzae*, anthrax, and others), the identification of mitochondrial inheritance, the role of transcription factors as regulators of embryonic development, the use of animal models to study human development and malformations (transgenic animals, *Xenopus*, zebrafish, non-human primates), neural plasticity and cognitive development, and the physical chemistry of the nervous system. Contributions of note have been made dealing with the properties of biological molecules and their structure and chemistry. Another major focus has been in the development and application of new technologies to study the biology of development at the subcellular, cellular, and organismic level; examples include confocal microscopic techniques to study cell and protein trafficking, laser-capture microscopy, diffusion tensor magnetic resonance imaging, and functional genomics to study global gene regulation.

2003 Honors and Awards

The following NICHD fellows received the 2003 NIH Fellows Awards for Research Excellence (FARE): Moonsuk S. Choi, Massimo De Martino, Yang Feng, Andre Fisahn, Renee Freedman, Michelle S. Horning, Ying Huang, Ho-Sang Jeong, Robert Kleta, Josh Lawrence, Erik Snapp, and Konstantina Vanevski. Shamik Parikh received the Pfizer Outstanding Fellow-in-Training Award.

Andreas Buonanno was elected member of the Latin American Academy of Sciences (ACAL). Y. Peng Loh gave the Plenary Lecture at the International Symposium on Chromaffin Cell Biology. Stanko Stojilkovic was elected as a member of the Serbian Academy of Sciences and Arts. Heiner Westphal was named an advisor to the German Government on issues regarding stem cells. Sergey Bezrukov was asked to serve as cochair of the Second International Conference on Noise in Biological, Biophysical, and Biomedical Systems. Juan Bonifacino cochaired the Workshop on Protein Trafficking: Molecular Mechanisms and Disease (see below) and was elected to the Counsel of the American Society for Cell Biology. Mary Dasso was co-organizer of the Third NICHD Symposium on the Eukaryotic Nucleus held at Berkeley Springs, West Virginia. Michael Cashel gave the plenary lecture at the 2003 Molecular Biology Division Symposium of the American Society for Microbiology. Douglas Fields delivered the Helen Wiltshire Walsh Lecture at the University of Miami, organized and chaired the Society for Neuroscience Symposium on Neuron-Glia Interactions, gave invited lectures at the IBRO Symposium in Prague, the European Glia Meeting at University College London, the Division of Neuroscience, University of Edinburgh, and Elan Pharmaceuticals in San Francisco, was editor-in-chief of *Neuron Glia Biology* and associate editor of *Neuroscience Letters*. Philip Nelson became a member of the Scientific Advisory Board of the United Cerebral Palsy Foundation. Amir Gandjbakhche gave invited lectures at the Conference in Laser and Electro-Optics in Baltimore and at the MIYI Conference in Tokyo, and chaired the Symposium on Inverse Problems in Biomedical Imaging in Vancouver (see below). Michael Lamb is scheduled to receive the James McKeen Cattell Fellow Award from the American Psychological Society in 2004. Jennifer Lippincott-Schwartz chaired the Molecular Membrane Biology Gordon Research Conference and the Keystone Conference on Cellular Dynamics and received the NICHD Health Award Merit; her paper “Photoactivatable GFP” is currently number one in the all time top papers for *Neuroscience*. Karel Pacak was appointed secretary general for the Eighth International Symposium on Catecholamines and other Neurotransmitters in Stress, held in Slovakia, cofounded and chairs the International Working Group on Endocrine Hypertension, and received the NICHD Staff Recognition Award. Brant Weinstein is associate editor of the journal *Zebrafish*.

Thus the Institute continues to mount an ambitious and highly regarded Intramural Program with a continuing increase in the quality and quantity of its scientific productivity. The challenge for the future is to maintain this desirable trend and build on current directions of scientific inquiry.

Owen M Rennert

Owen M. Rennert, M.D.
Scientific Director
National Institute of Child Health
and Human Development

INTRODUCTION

RESEARCH FOCUS OF THE LABORATORIES AND BRANCHES

1. BONE AND EXTRACELLULAR MATRIX BRANCH

Joan C. Marini, MD, PhD, Chief

The newly formed Bone and Extracellular Matrix Branch (BEMB) has begun to conduct research on the extracellular matrix of bone and on diseases resulting from defective matrix. Primary defects of matrix, such as the genetic disorder osteogenesis imperfecta, result in severe osteoporosis and life-long disability. Laboratory studies aimed at understanding the defects' genetic etiology and molecular and cellular mechanisms will yield insight into normal skeletal functioning. The branch's clinical protocols focus on improved clinical diagnosis and treatments.

2. CELL BIOLOGY AND METABOLISM BRANCH

Juan Bonifacino, PhD, Chief

The Cell Biology and Metabolism Branch (CBMB) conducts studies on the mechanisms of intracellular protein trafficking and organelle biogenesis, the biology of metal metabolism, the adaptive responses to environmental stresses, and the regulation of the cell cycle during oogenesis. The CBMB's outstanding microscopy facilities permit the use of state-of-the-art techniques such as fluorescent imaging of cells in real time, photobleaching, fluorescence resonance energy transfer, fluorescence correlation spectroscopy, and image analysis in the study of cell structure and dynamics. In addition, the CBMB maintains facilities for cell microinjection and micromanipulation, for automated DNA sequencing, and for work with bacteria, yeast, *Drosophila melanogaster*, and mammalian cells. Members of CBMB apply knowledge gained from the study of basic cell biological problems to the elucidation of the causes of human diseases, including disorders of lysosome-related organelles, iron overload, and neurodegeneration.

Over the past year, the *Section on Intracellular Protein Trafficking*, led by **Juan Bonifacino**, discovered critical components of the molecular machinery involved in protein secretion and transport to different compartments within the cell. The group has identified proteins and interactions that determine transport to endosomes and lysosomes, including a novel protein complex that is defective in mouse models of the pigmentation and bleeding disorder Hermansky-Pudlak syndrome.

The *Unit on Protein Biogenesis*, led by **Ramanujan Hegde**, has studied the alterations in the biogenesis of the prion protein (PrP) in the endoplasmic reticulum (ER) that lead to the development of neurodegenerative disease. The group has made significant progress toward a molecular description of PrP translocation, demonstrating how key steps during the process can be modulated to influence the generation of potentially neurotoxic forms of PrP. These advances are beginning to illuminate the entry of secretory and membrane protein substrates into the mammalian secretory pathway.

Catherine Jackson's group, the *Unit on GTPase Regulation of Membrane Traffic*, has identified proteins that play important roles in protein trafficking through the Golgi apparatus, in particular binding partners of activators of the Arf GTPase, a central regulator of organelle structure and function. The interactions shed new light on the mechanisms of trafficking through the secretory pathway.

The *Unit on Cell Cycle Regulation*, led by **Mary Lilly**, has used genetic approaches to identify genes that regulate the cell cycle during gametogenesis in *Drosophila*, providing unique insights into the poorly understood pathways that drive early meiotic progression and oocyte development in metazoans.

The *Section on Organelle Biology*, led by **Jennifer Lippincott-Schwartz**, has continued the development of novel fluorescence imaging tools that promise to revolutionize the analysis of protein dynamics within cells. Some of these tools are variants of the green fluorescent protein (GFP) that show greatly increased fluorescence after activation and allow the analysis of the movement and fate of proteins within the cell. The group is applying the technology to the study of cell division, lysosome biogenesis, and protein secretion.

Tracey Rouault's group, the *Section on Human Iron Metabolism*, studies the regulation of iron metabolism, in particular mechanisms of iron cluster assembly and enzymes and chaperones involved in iron cluster assembly in mitochondria and the cytosol. The group has also analyzed the physiological roles of two iron-regulatory proteins (IRP1 and IRP2) in iron metabolism and in the function of the central nervous system. Defects in IRP2 in mice were found to cause a progressive neurodegenerative disorder.

Under the direction of **Gisela Storz**, the *Section on Environmental Gene Regulation* studies the mechanisms by which cells defend against oxidative stress. The group conducted analyses of key regulators of the cellular responses to hydrogen peroxide in bacteria and yeast. Another area of research is the identification and characterization of untranslated, regulatory RNAs. Systematic screens for noncoding RNA genes in *E. coli* have resulted in the identification of 24 new noncoding RNAs.

3. DEVELOPMENTAL ENDOCRINOLOGY BRANCH

Carolyn Bondy, MD, Chief

The Developmental Endocrinology Branch (DEB) investigates human growth and development. **Jeffrey Baron** and his colleagues in the *Unit on Growth and Development* study the cellular and molecular mechanisms governing bone growth and development and apply their findings to a deeper understanding of human skeletal disorders. They have shown that estrogen promotes epiphyseal fusion by accelerating the normal process of growth plate senescence and that growth hormone treatment modestly increases the final height of children with non–GH-deficient short stature.

The *Section on Endocrinology and Genetics*, led by **Constantine Stratakis**, focuses on genetic and molecular disorders of the adrenal cortex, with emphasis on those that are hereditary and associated with multiple tumors in other endocrine glands. The section's work has shown that many sporadic adrencortical adenomas harbor either mutations of the PRKAR1A gene or PKA functional abnormalities, strongly implicating this novel pathway in adrenal tumorigenesis.

Groups led by **Carolyn Bondy**, the *Section on Growth and Metabolism*, the *Section on Women's Health Research*, and the *Unit on Turner's Syndrome*, integrate basic and clinical research to elucidate genetic and physiological mechanisms governing male and female development. One group demonstrated that young, lean women with Turner's syndrome have an atherogenic lipid profile with increased risk for diabetes compared with age- and BMI-matched women suffering from premature ovarian failure, suggesting that haploinsufficency for X-chromosome genes may explain the increased risk for coronary heart disease in this syndrome and perhaps in 46XY men as well.

Jack Yanovski's group, the *Unit on Growth and Obesity*, has identified a melanocortin 3 receptor variant with diminished signal transduction that is significantly associated with adolescent obesity, especially in African Americans, and that may help explain the propensity for obesity in this group.

Lawrence Nelson's group, the *Unit on Gynecologic Endocrinology*, focuses on premature ovarian failure (POF), which affects 1 percent of all women under age 40. This past year, the group demonstrated that measuring adrenal antibodies is an effective screening method with which to detect asymptomatic autoimmune adrenal insufficiency in young women with spontaneous POF.

Led by **Greti Aguilera**, the *Section on Endocrine Physiology* focuses on the molecular mechanisms controlling neuroendocrine components of the stress response, especially the hypothalamic peptide corticotropin releasing hormone (CRH), and the V1b type vasopressin (VP) receptor, and has demonstrated that cAMP-dependent pathways stimulate CRH expression at the level of transcription, mRNA stability, and mRNA translation, and that these effects are modulated by distal downstream elements in the CRH gene. The section also characterized mechanisms regulating transcription and translation of V1b VP receptors and demonstrated that deficient mineralocorticoid secretion and renal insensitivity to vasopressin contribute to alterations in fluid and electrolyte homeostasis induced by endotoxemia.

4. ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH

Kevin Catt, MD, PhD, Chief

The Endocrinology and Reproduction Research Branch (ERRB) investigates the molecular and cellular mechanisms of reproductive hormone action, the structure-function properties and signaling pathways of peptide hormone receptors, and the roles of phosphorylation and sulfonation in metabolic regulation and signal transduction.

Tamas Balla leads the *Unit on Molecular Signal Transduction,* whose studies on phospholipid signaling revealed that PH domains, which bind to phosphoinositides, regulate signaling proteins and recognize inositol lipids in the context of other protein components, accounting for the domains' highly specific inhibition of PI(3,4,5)P3-mediated cellular responses. Localization of PH domains that recognize PI(4)P, the lipid product of PI 4-kinases, is determined by both PI(4)P and Golgi proteins. Investigations of these PH domains revealed that type-III PI 4-kinases are largely responsible for PI(4)P synthesis in the plasma membrane.

Kevin Catt's group, the *Section on Hormonal Regulation,* investigates the structure-function properties and signaling pathways of peptide hormone receptors and the roles of autocrine regulation and G protein switching in episodic GnRH secretion. Angiotensin AT_1 receptors and GnRH receptors were found to activate EGF receptor–mediated MAP kinase signaling in hepatic and neuronal cells via liberation of HB-EGF. In addition to such GPCR-induced transactivation of a receptor tyrosine kinase, the group identified a reciprocal mechanism in which EGF receptor activation stimulates AT_1 receptor phosphorylation.

Studies conducted by the *Molecular Endocrinology Section,* which is led by **Maria Dufau**, on orphan receptor–mediated silencing of LH receptor gene transcription have demonstrated that COUP-TFI/EAR3 bound to DNA perturbs communication between the Sp1/Sp3 complex and TFIIB, reducing recruitment of RNA Pol II. This mechanism operates in repressive/inductive receptor states during the ovarian cycle. A novel gonadotropin-regulated RNA helicase (GRTH/Ddx25) was found in Leydig and germ cells. Testosterone increases GRTH expression at the transcriptional level and promotes utilization of one of three ATG codons in both cell types.

The *Section on Metabolic Regulation*, led by **Kuo-Ping Huang**, discovered that neurogranin, a neuronal calmodulin (CaM)-binding protein, enhances hippocampus-dependent learning and memory by promoting calcium-mediated signaling. Neurogranin modulates synaptic responses by sequestering CaM and regulating free Ca^{2+} and Ca^{2+}/CaM. NMDA receptor–mediated calcium influx triggers the stimulation of PKC, Ca^{2+}/CaM kinases, adenylyl cyclases, and NO synthase. The phosphorylation and oxidation of neurogranin participate in the regulation of Ca^{2+}- and Ca^{2+}/CaM-dependent neuronal signaling in the hippocampus to promote the enhanced synaptic plasticity that underlies learning and memory.

Stanko Stojilkovic's group, the *Section on Cellular Signaling,* studies nucleotide-gated receptor channels and cyclic nucleotide–controlled channels expressed in anterior pituitary cells and their roles in calcium signaling and hormone secretion. The group identified residues critical for nucleotide binding to recombinant nucleotide receptors as well as the impact of receptor-ATP affinity on C-terminal domain–controlled receptor desensitization. The group also characterized dependence of guanylyl cyclase activity on intracellular calcium, nitric oxide, and phosphorylation by protein kinase C and of cyclic nucleotide–gated channel activity on basal and receptor-stimulated adenylyl/guanylyl cyclases.

Charles Strott's group, the *Section on Steroid Regulation,* studies the SULT2 subfamily of cytosolic sulfotransferases that sulfoconjugate DHEA (SULT2A1), pregnenolone (SALT2B1a), and cholesterol (SALT2B1b). SULT2B1b is exclusively expressed in keratinocytes, where it is confined to epidermal granular cells, and in platelets. SULT2B1a is selectively expressed in the central nervous system, where pregnenolone sulfate is important in learning and memory. Comparison of the 3-D structures of SULT2B1 isoforms and SULT2A1 has yielded new insights into the molecular basis of substrate selectivity.

5. HERITABLE DISORDERS BRANCH

Joan C. Marini, MD, PhD, Chief, June 1995 to September 30, 2003

Constantine Stratakis, MD, DSc, Acting Chief from October 1, 2003

Joan Marini's research program on osteogenesis imperfecta (OI) within the Heritable Disorders Branch (HDB) has focused on the effects of the bisphosphonate drugs in both children and mice with OI and on a collaborative study of an interesting subset of OI patients who have both OI and Ehlers-Danlos syndrome. Treatment of the Brtl mouse model for OI, previously generated by Marini's group, the *Section on Connective Tissue Disorders*, and of children with types III and IV OI resulted in increased bone density. However, biomechanical tests of murine femurs did not show improvement in the brittleness of the bone. For the OI/EDS cases, the laboratory defined a five-exon region of type I collagen in which mutations cause EDS symptoms by retention of the N-propeptide. As of October, 2003, this research was continued in the Bone and Extracellular Matrix Branch.

Denny Porter's group, the *Unit on Molecular Dysmorphology,* studies children with disorders of cholesterol synthesis, including the Smith-Lemli-Opitz syndrome, and generates murine models for those disorders. The carrier frequency for SLOS in African Americans was found to be 0.73 percent, similar to the frequency in Caucasians, and phenotypic variants are being sought. The group has generated a new mouse model for defects in the enzymatic step immediately preceding the SLOS defect and identified a patient with the novel lathosterolosis malformation syndrome. Comparison of the lathosterolosis mouse with the SLOS mouse model will yield important insights into the hedgehog signaling pathway.

The investigations of **Janice Chou**'s laboratory, the *Section on Cellular Differentiation,* have focused on glycogen storage disease and G6PT. A structure-function study of the G6PT used an adenoviral vector–mediated expression system and found that most codon mutations completely abolish microsomal G6P uptake activity while others partially inactivate uptake. Mutations have also been characterized within the signature motif shared by G6PT and a family of transporters of phosphorylated metabolites, showing that the motif is a functional element required for microsomal transport. Using its murine knockout for G6PT, the group has demonstrated that the neutropenia associated with the disorder is directly caused by the loss of G6PT activity.

The *Section on Developmental Genetics*, led by **Anil Mukherjee**, focuses on heritable autoimmune and neurodegenerative disorders and is engaged in a pilot study to determine if cystagon is an effective treatment for infantile neuronal ceroid lipofuscinosis (INCL). The group is using a mouse model of INCL to understand the molecular mechanisms of the disease. The researchers have recently demonstrated that uteroglobin is a repressor of PGD_2 receptor–mediated *COX-2* gene expression and an inhibitor of prostaglandin $F_{2\alpha}$ receptor–mediated activation of NF-kappaB expression and that uteroglobin knock-out mice are predisposed to lung tumors when exposed to NNK commonly present in cigarette smoke.

Ida Owens's group, the *Section on Genetic Disorders of Drug Metabolism,* focuses on the molecular biology, regulation, and biochemistry of UDP-glucuronosyltransferase enzymes and has discovered that the isozymes require phosphorylation that is regulated through signaling. The section also has evidence that phospho-groups play an operational role in catalysis rather than causing the typical conformational change in protein structure. The bilirubin-metabolizing isozymes are segmentally distributed through the GI system and may play an important role in limiting GI absorption of polyphenols.

6. LABORATORY OF CELLULAR AND MOLECULAR BIOPHYSICS

Joshua Zimmerberg, MD, PhD, Chief

Using systems ranging in complexity from well-defined molecular composition and structure to human tissue to investigate the physico-chemical basis of molecular, physiological, and pathological processes, the Laboratory of Cellular and Molecular Biophysics (LCMB) develops novel, noninvasive technologies to probe the processes' physical and chemical parameters. The research includes the physical chemistry of gas phase ions, polymer organic chemistry, membrane biochemistry, electrophysiology, cell biology, parasitology, immunology, tissue culture, virology, and HIV pathogenesis.

Joshua Zimmerberg's group, the *Section on Membrane and Cellular Biophysics,* studies the mechanisms of membrane remodeling in viral and parasite infection, exocytosis, and apoptosis. Using the physics of continuum bilayers and direct observations of biological fusion, analytical and numerical calculations of membrane energetics, and experiments on phospholipid bilayers, purified proteins, cell expression systems, purified organelles, and cell surface complexes, the section has elucidated the energetics of membrane tubule shape dynamics and permeability and membrane permeability during fusion. Studies of the physiological and pathogenic events of fertilization and viral infection, e.g., those involving SNAREs, calcium, and exocytosis, have led to novel uses of viral fusion proteins for gene therapy and to a new model for the cycle of exocytosis and endocytosis.

The *Section on Membrane Biology*, led by **Leonid Chernomordik**, studies the mechanistic pathway of membrane fusion. Over the past year, the section developed a technique for measuring the complete kinetics of the lipid mixing, finding evidence that distinct fusion machines generate distinct phenotypes. In another study, the researchers found that the widely used cell-penetrating peptides that readily deliver themselves, conjugated proteins, and nucleic acids into living cells enter cells by endocytosis rather than by direct translocation through the membrane, as is often hypothesized.

Leonid Margolis's group, the *Section on Intercellular Interactions,* found that human lymphoid tissue *ex vivo*, a system developed in the LCMB, supports productive infection with different types of HIV-1 isolates, dissemination of virus throughout the tissue, depletion of $CD4^+$ T cells, release of virus into the media, lymphocyte apoptosis, and a functional immune response, thus providing a unique way to study HIV tissue pathogenesis. In this system, both activated and nonactivated $CD4^+$ T cells support productive infection; a combination of productive infection and activation is sufficient for T cell apoptosis; productive HIV infection is sufficient to deplete infected $CD4^+$ T cells; and herpes virus 6, an HIV-1 copathogen, infects both memory and naïve T cells, profoundly influencing the physiology of the immune responses and the replication of different HIV-1 variants.

The *Section on Metabolic Analysis and Mass Spectrometry*, led by **Alfred Yergey**, applies the physical chemistry of gas phase ions to research in structural biology. Studies include mapping of picomolar quantities of peptides extracted from proteins digested *in situ* from electrophoretically separated proteins; obtaining partial peptide sequences at sub-picomolar sensitivities to facilitate the construction of nucleotide probes; and mapping epitopes of femtotomolar quantities of proteins isolated by noncovalent interactions with antibodies.

Andreas Chrambach's group, the *Section on Macromolecular Analysis,* pursues the improvement of analytic and preparative electrophoresis with biological and methodological aims. The biological interest is oriented toward the idea that the present focus on an inventory of gene products will decline in the future. Correspondingly, interest will shift to the development of an inventory of the post-translational forms of macromolecules, intermolecular complexes, and subcellular particles that constitute the building blocks of biological reality. Methodologically, the section attempts to upgrade the common practice of gel electrophoresis through the following development projects: (1) theory and computer programs for deriving information from mobility regarding physical properties of analytes and polymer networks and for predicting resolution; (2) resolution of currently unresolved or poorly resolved analyte types such as subcellular-sized particles and the size- and charge-isomeric forms of peptides and their complexes; (3) detergent and miniaturized gel methods suitable for native membrane proteins and their complexes of a low level of occurrence; and (4) automated analytic and preparative gel electrophoresis methods.

Within LCMB, the *NASA/NIH Center for Three-Dimensional Tissue Culture*, a pan-NIH facility directed by **Joshua Zimmerberg**, with deputy directors **Leonid Margolis** and **Paul Blank**, provides NIH researchers with an opportunity to develop new model systems for diseases whose pathology cannot be reproduced in growing cells in monolayer culture. Several NASA-designed rotating wall vessels, which culture cells under minimal shear forces in a well-oxygenated medium under conditions that mimic microgravity, are available, along with experienced technicians to test tissues, primary cell cultures, and cell lines under conditions that seem to facilitate cell-cell interactions and promote differentiation. Extensive consultations with a group seminar determine the applicability of Center resources to the aims of the interested principal investigator (PI). A PI with successful pilot projects can apply for Center funding for salary, equipment, and consumables. Projects are fully mature when PIs continues the work with their own funding. Given the surprise finding of immunodysfunction in human lymphoid tissue in culture and the known findings of immunodysfunction in astronauts after space flight, Dr. Zimmerberg is also a NASA flight PI on the International Space Station (ISS).

7. LABORATORY OF CELLULAR AND MOLECULAR NEUROPHYSIOLOGY

Mark L. Mayer, PhD, Chief

Ionotropic glutamate receptors (GluRs) are molecular pores that mediate signal transmission at the majority of excitatory synapses in the mammalian nervous system. Given their essential role in normal brain function and development and increasing evidence that dysfunction of GluR activity mediates multiple CNS diseases as well as damage during stroke, the Laboratory of Cellular and Molecular Neurophysiology (LCMN) is directing considerable effort to the analysis of GluR function at the molecular level, with the goal of obtaining atomic-resolution structural data. The data will provide a framework in which to design experiments to define the mechanisms underlying ligand recognition and gating, which, in turn, will allow the development of subtype-selective antagonists and allosteric modulators with novel therapeutic applications.

The ionotropic glutamate receptors in humans are encoded by seven gene families named after the ligands that were first used to identify the major subtypes on a functional basis: AMPA, kainate, and NMDA. The recent crystallization of the ligand-binding cores of an AMPA receptor subunit and a related bacterial receptor from the photosynthetic bacterium syncheeocystis PCC 6803 has revealed the molecular mechanisms underlying the binding of agonists and antagonists and has provided insight into the mechanisms of activation and desensitization. During the past year, the LCMN has undertaken similar structural studies on members of the kainate receptor gene family as well as on the delta receptor orphan subunits. The structure of a GluR6 ligand-binding core complex with glutamate was solved by SeMET MAD crystallography, and two additional glutamate complexes in different crystal forms were solved by molecular replacement. In addition, the LCMN used Fourier difference techniques to solve the structures of complexes with the high-affinity agonists quisqualate and 4-methylglutamate.

The LCMN's functional studies over the same period had two goals: an analysis of the mechanism of partial agonist action and the identification of the contacts in the dimer interface of the GluR2 AMPA receptor ligand-binding core responsible for maintaining the active state of the receptor, establishing that 5-substituted willardiines act as partial AMPA receptor agonists, for which the extent of activation and desensitization are inversely related to the size of a 5-position substituent. Crystallographic studies revealed that, as a result of steric hindrance, large 5-substituents prevent domain closure of the agonist binding core to the same extent as produced by full agonists. As a correlate, single-channel studies revealed that full and partial agonists activate the same subconductance states, but with different relevant occupancy. Studies on the GluR2 ligand-binding core dimer interface used the crystal structure as a basis for designing mutants to test the role of ion pair interactions, hydrogen bonds, and van der Waals contacts of hydrophobic patches in maintaining the active state. The results are in excellent agreement with those predicted by the crystal structure and reinforce our earlier proposal that such contacts are a key structural element in the gating mechanism.

8. LABORATORY OF CELLULAR AND SYNAPTIC NEUROPHYSIOLOGY

Chris McBain, PhD, Chief

The Laboratory of Cellular and Synaptic Neurophysiology (LCSN) uses the techniques of neurophysiology, molecular biology, and cell biology to study signaling mechanisms related to the development, physiology, and pathophysiology of the mammalian central nervous system. LCSN researchers study receptors, ion channels, and signaling mechanisms in preparations that range from isolated cells to highly ordered neural networks from both wild-type and transgenic animals in physiological and pathophysiological conditions. Problems under analysis concern mechanisms of short- and long-term plasticity of synaptic transmission, neurotrophin regulation of excitability and development, differential targeting of synaptic receptors, pathophysiological processes in clinically relevant neuronal migration disorders, ion channel regulation of development and excitability, drug action at a variety of voltage- and ligand-gated receptors, and synaptic and network mechanisms underlying sensory processing and memory formation, including coding mechanisms involving the oscillatory interactions of ensembles of interneurons in the insect antennal lobe.

Chris McBain's group, the *Section on Cellular and Synaptic Physiology,* achieved the extensive characterization of Ca-permeable, GluR2-lacking AMPA- and NMDA-receptors at hippocampal inhibitory interneuron synapses; the elucidation of the time course of electrotonically overlapping but kinetically distinct synaptic conductances on inhibitory interneuron dendrites; and the roles of voltage-gated conductances in the generation of muscarinic receptor– and kainate receptor–induced hippocampal gamma oscillatory activity.

Bai Lu and his colleagues in the *Section on Neural Development and Plasticity* discovered the function of the pro-domain of the BDNF protein in synaptic targeting and activity-dependent local secretion of BDNF. He also demonstrated activity- and Ca^{2+}-dependent regulation of the BDNF receptor TrkB and of TrkB endocytosis. These findings provide insight into how diffusible BDNF elicits local and synapse-specific modulation. In addition, Lu's laboratory identified a novel form of synaptic plasticity that is independent of Ca^{2+} influx but dependent on a mitochondrial Na^+-Ca^{2+} exchanger.

Dax Hoffman generated and began characterizing mutated potassium channel constructs expressed in organotypic slice cultures. In addition, his group, the *Molecular Neurophysiology and Biophysics*

Unit, has expanded a dominant negative line of transgenic mice expressing a pore mutation in Kv4.2, a potassium channel subunit theorized to be important in regulating dendritic signal propagation in the hippocampus.

The past year also saw the start of a new laboratory, the *Unit on Sensory Coding and Neural Ensembles*, led by **Mark Stopfer**. He combines imaging, patch clamp physiology, and behavioral analysis to investigate synaptic and network mechanisms underlying sensory processing and memory formation, including coding mechanisms involving the oscillatory interactions of ensembles of interneurons in the insect antennal lobe.

9. LABORATORY OF CLINICAL GENOMICS

Owen M. Rennert, MD, Chief

The Laboratory of Clinical Genomics (LCG) focuses on the interface between biological and clinical research dealing with the processes of development/differentiation and diseases in which these phenomena have pathological manifestations.

The *Section on Developmental Genomics*, led by **Owen Rennert**, has focused on functional genomic studies of spermiogenesis, molecular cloning, and the characterization of gene networks during spermatogenesis; genetic regulation of early gonadal and germ cell development; and disease-causing mutations of the LH receptor and their phenotypic manifestations. Using SAGE and QPCR analysis to study global gene expression at different stages of spermiogenesis, the section has documented that a *core set of genes* is required in all germ cells and their progenitors for basic biological functions. In addition, the researchers identified a *distinct set of genes* that are expressed at cell-specific stages (mitotic, meiotic, and post-meiotic) of spermatogenesis and require their concerted action. The investigators have also documented that a number of X-linked genes escape transcription inactivation during meiosis and in the post-meiotic stage. During the past year, research focused on the characterization of two X-linked genes, *Tex13* and *Scml2*, documenting the transcription of both sense and antisense messages in different stages of germ cell differentiation. In a collaborative study, gene transcription is under investigation with both SAGE and microarray hybridization during the period E10.5 through 17.5 in the mouse, during which sexual dimorphism of the mouse gonad begins with expression of the *Sry* gene, through the period when differentiation into male and female gonads occur with formation of the gonocyte and oocyte.

The section has identified more than 70 percent of the mutations of the LH receptor associated with familial male-limited precocious puberty and Leydig cell hypoplasia, providing a national resource for the molecular diagnostic studies that establish these clinical diagnoses. With respect to transfected cell expression systems, the section is investigating trafficking and processing of the LH receptor and the downstream "expression" events. In addition to these clinically related studies, the researchers have investigated the role of thrombophilia in genetic susceptibility to a variety of complex disorders, specifically those associated with pseudotumor cerebri. They have identified a polymorphism in coagulation factor V as an etiologic factor in the condition idiopathic intracranial hypertension that lends support to the postulation that this condition arises as a consequence of a defect in CSF reabsorption. Most recently, the group has applied microarray analysis to study the role of the tumor suppressors $p75^{NTR}$ and U94 in human prostate cancer.

During the past year, the *Unit on Pediatric Genetics*, led by **Steven Kaler**, has investigated two groups of disorders, the X-linked recessive disorder called Menkes Kinky Hair disease and the platelet abnormality known as Bernard-Soulier syndrome. In the case of Menkes Kinky Hair disease, the unit has investigated the downstream gene-expression consequences of a defect in the copper-ATP transport system in both clinical autopsy material and a yeast knock-out strain. The investigators studied a child who had the velo-cardio-facial syndrome with thrombophilia due to haploinsufficiency of chromosome 22q11. Their research led to the delineation of platelet glycoprotein GPIb-IX complex deficiency.

10. LABORATORY OF COMPARATIVE ETHOLOGY

Stephen Suomi, PhD, Chief

The Laboratory of Comparative Ethology (LCE) carries out a program of basic biobehavioral research investigating cognitive, social-emotional, and biological development in humans and nonhuman primates. The researchers study both genetic and environmental factors and their multiple interactions from a comparative perspective in order to characterize the developmental trajectories of individuals across a broad range of species, populations, and settings. Field and laboratory-based studies of biobehavioral development in nonhuman primates are designed specifically to facilitate comparisons with findings from long-term prospective investigations of human infants and their families as well as with data obtained by neuroscience techniques, thereby promoting translational analyses. Most studies employ longitudinal designs in order to address basic issues of continuity versus change and the relative stability of individual differences throughout development; many investigations rely on a variety of both behavioral and biological measures reflecting multiple levels of analysis in order to examine patterns of covariance among measures both within and between levels of analysis. To identify particularly recurrent or unusual patterns and evaluate their consequences with respect to established norms, the laboratory places considerable emphasis on characterizing and understanding normative patterns of development.

Stephen Suomi and his colleagues in the *Section on Comparative Behavioral Genetics* investigate biological and behavioral development in selected nonhuman primate species by focusing on interactions between genetic and environmental factors that affect the course of individual developmental trajectories throughout the lifespan.

John Newman's group, the *Unit on Developmental Neuroethology,* uses neuroscience techniques to study brain mechanisms involved in the production of various types of primate vocalizations, to examine subtle acoustical differences in characteristic calls, and to investigate the calls' function in several primate species.

Marc Bornstein's group, the *Child and Family Research Section,* examines cognitive, language, and social-emotional development in human infants and children, with special emphasis on the relationships among early attentional processes, social stimulation from and interactions with caregivers, and subsequent cognitive and social behavioral capabilities.

Michael Lamb heads the *Section on Social and Emotional Development,* which studies the effects of different types of care-giving practices on human infant and toddler social and emotional development and cognitive competence. The team devotes particular attention to longitudinal approaches that transcend infancy, childhood, and adolescence and to process-oriented studies of interactional styles, memories, and social perceptions.

11. LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY

John B. Robbins, MD, Chief

The Laboratory of Developmental and Molecular Immunity (LDMI) studies the pathogenesis of and immunity to bacterial diseases, especially those of infants and children, with the overall objective of developing vaccines for their prevention. Given that almost all pathogens of healthy individuals are inhabitants of humans only, the LDMI brings its investigational vaccines to clinical evaluation for safety, immunogenicity, and, ultimately, efficacy, as soon as possible. Research at LDMI is guided by the principle that "the proper study of mankind is man."

Members of the LDMI pioneered the development of a new generation of vaccines in which specific antigenic capsular polysaccharides are chemically conjugated to highly immunogenic but nonspecific proteins. The new conjugate vaccines, which confer T-cell dependence and booster responses to polysaccharide antigens, have been singularly successful, as exemplified by the *H influenzae* type b conjugate vaccine. *H. influenzae* type b meningitis (the most common cause of acquired mental retardation) has been virtually eliminated wherever the vaccine has been used. The principles and methods developed for this vaccine have now yielded conjugate vaccines against *Salmonella typhi*, nontyphoidal *Salmonellae*, *Shigellae*, and *Vibrio cholerae*, which are being field tested. By producing a nontoxic mutant shigella-like toxin and conjugating it to the capsular polysaccharide of *E. coli* O157, LDMI researchers produced an experimental vaccine against this pathogen, which causes the often fatal hemolytic uremic syndrome, especially in small children. A conjugate vaccine against *Staphylococcus aureus,* the cause of many hospital-acquired infections, has been tested successfully in hemodialysis patients, who are also at increased risk of infection by this pathogen. In an effort to produce an anthrax vaccine with fewer side effects than the currently available vaccine, a recombinant vaccine against *Bacillus anthracis*, produced on an industrial scale in collaboration with researchers at NIDDK, has been shown to elicit comparable levels of neutralizing antibody in mice and is now under review by the FDA. Work is in progress to also produce a conjugate vaccine against *Borrelia burgdorferi,* the causative agent of Lyme disease.

12. LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

Owen M. Rennert, MD, Acting Chief

The goal of the Laboratory of Developmental Neurobiology (LDN) is to understand the molecular and cellular mechanisms underlying regulation of the development and function of the nervous system. Major topics of interest include the mechanisms of processing, intracellular trafficking, and secretion of neuropeptides and hormones; the molecular machinery of secretory vesicle biogenesis and the presynaptic release process; circadian biology with emphasis on the pineal gland and synthesis of melatonin; and the coupling between action potentials, neurotransmitters, neurohormones, neurotrophic factors, and regulation of gene expression.

Peng Loh's group, the *Section on Cellular Neurobiology,* studies intracellular protein trafficking and granule biogenesis in neuroendocrine and exocrine systems. Her group recently uncovered the molecular basis for the intracellular missorting of mutant proinsulins found in hyperproinsulinemia patients and has shown a major defect in zymogen secretory granule formation and maturation in the exocrine pancreas of a mouse model of Smith-Lemli Opitz syndrome. In collaboration with Joanna Hill, the section showed the importance of carboxypeptidase E in a mouse lacking this enzyme, not only for neuropeptide processing and trafficking but also in the early development of the nervous system.

To identify genes that are expressed at elevated levels at night and genes that are selectively expressed in the pineal gland, **David Klein**'s group, the *Section on Neuroendocrinology,* has applied microarray technology to the analysis of control of pineal function. The section also began a global analysis of rhythmic and pineal-specific genes, with the intention of identifying common critical regulatory elements. In addition, the section led an NICHD initiative to develop a commercial microarray tool for analyzing gene expression in the zebrafish.

Using biochemical and morphologic techniques in primary cell cultures of fetal mouse spinal cord, the *Section on Cell Biology*, led by **Elaine Neale**, studies synaptic function in neurons as affected by tetanus and botulinum neurotoxins. The results have implicated recycling synaptic vesicles in the neuronal uptake of botulinum neurotoxins serotypes A and E and further suggest that the two toxins are trafficked differently, perhaps underlying the much longer duration of action of BoNT A.

Led by **Andres Buonanno**, the *Section on Molecular Neurobiology* found that distinct neuregulins, a family of growth and differentiation factors that regulate the expression of glutamate neurotransmitter receptors and that were recently implicated in schizophrenia, are expressed in diverse neuronal populations during development and are differentially targeted to either axon terminals or dendrites, suggesting that these related factors have nonoverlapping functions in brain.

Philip Nelson's group, the *Section on Neurobiology*, studies activity-dependent synaptic plasticity during development. Both pre- and post-synaptic mechanisms are involved in the Hebbian response to synapse activation that is captured in the multicompartment tissue culture model of the neuromuscular junction that the section developed. An antagonistic interaction between protein kinase A and C regulates synaptic stability, in particular the loss or retention of the acetylcholine receptor (AChR). PKA stabilizes and PKC destabilizes the receptor. The section also demonstrated antagonistic effects of kinase action on the presynaptic transmitter release and showed that glia-derived neurotrophic factor (GDNF) increases the stability of the AchR while minimally affecting the synthesis or membrane insertion of the receptor.

13. LABORATORY OF GENE REGULATION AND DEVELOPMENT

Alan G. Hinnebusch, PhD, Chief

Five of the eight groups in the Laboratory of Gene Regulation and Development (LGRD) study mechanisms of gene regulation, chromosome condensation and segregation, and the transposition of retroelements in yeasts.

The *Section on Nutrient Control of Gene Expression*, led by **Alan Hinnebusch**, studies the transcriptional and translational control of amino acid biosynthetic genes in response to nutrient availability. Recently, the section described the molecular interactions of a multifactor complex of translation initiation factors with the 40S ribosome and discovered that the initiation factor 2 (eIF2) kinase GCN2 is regulated by the TOR nutrient signaling pathway.

Thomas Dever's group, the *Section on Protein Biosynthesis*, is characterizing the structure and function of several translation initiation factors (eIFs) and the molecular principles of kinase-substrate recognition. The section recently determined that residues remote from the phosphorylation site contribute to substrate recognition by the eIF2 kinases. It also found that a GTP-regulatory switch governs the affinity of eIF5B for the ribosome.

Rohinton Kamakaka's group, the *Unit on Chromatin and Transcription*, studies gene silencing in yeast. In addition to recently purifying and characterizing the Sir2 protein complexes, the group demonstrated a role for histone Htz1 in cell cycle progression and silencing. The researchers also performed a genome-wide screen for proteins that can block the spread of silencing, identifying proteins involved in chromatin remodeling that possess this blocking ability.

Alexander Strunnikov's group, the *Unit on Chromosome Structure and Function*, is studying proteins controlling mitotic chromosome segregation fidelity, particularly condensin and cohesin complexes, involved in chromosome condensation and sister chromatid cohesion, respectively. Recently, the unit reconstituted an elementary sister chromatid cohesion reaction from purified components *in vitro*. The researchers also uncovered the molecular basis of interplay between topoisomerase II and condensin in chromosome condensation.

Led by **Henry Levin**, the *Section on Eukaryotic Transposable Elements* is analyzing an LTR-retrotransposon that shares many structural features with mammalian retroviruses. To define the molecular mechanisms of reverse transcription, the importing of particles into the nucleus, and the transsposon's preference for integration at pol II promoters, the group is studying the transposon Tf1, which propagates in *S. pombe*. This year, the section reported, e.g., that the Gag-like protein of Tf1 forms the coat of the virus-like particles and that the integration of Tf1 occurs preferentially at sites adjacent to pol II promoters.

Chi-Hon Lee's group, the *Unit on Neuronal Connectivity*, investigates the mechanism by which cadherin-based adhesion specifies the synaptic connections in the *Drosophila* visual system. The group developed a genetic method for the single-cell mosaic analysis at different developmental stages and, with it, demonstrated that neuronal cadherin regulates the initial target recognition process as well as the maintenance of the afferent-target interaction.

The anuran *Xenopus laevis* serves as a model system for two groups. Under **Yun-Bo Shi**, the *Section on Molecular Morphogenesis* studies the gene-regulatory mechanisms involving the thyroid hormone receptor (TR) that establish the developmental program of metamorphosis. Using transgenesis and chromatin immunoprecipitation on tadpole tissues, the section showed that gene activation by TR involving histone acetylation is essential for metamorphosis and that the TR-regulated matrix metalloproteinase stromelysin-3 facilitates apoptosis during intestinal remodeling through ECM remodeling.

Mary Dasso leads the *Section on Cell Cycle Regulation*, which studies the Ran GTPase and the SUMO family of ubiquitin-like proteins. The researchers have demonstrated that the spindle checkpoint is responsive to Ran-GTP and that Ran plays an important role in the metaphase-anaphase transition. They have also shown that topoisomerase-II is a major mitotic substrate of SUMO-2 conjugation and that inhibition of SUMO conjugation causes defects in chromatid separation.

14. LABORATORY OF GENOMIC INTEGRITY

Roger Woodgate, PhD, Chief

Scientists within the Laboratory of Genomic Integrity (LGI) study the mechanisms by which mutations are introduced into damaged DNA. Recent studies have shown that many of the proteins long implicated in the mutagenic process are, in fact, low-fidelity DNA polymerases that can replicate by moving past damaged DNA in a process termed translesion DNA synthesis (TLS). Humans with defects in one such polymerase, pol eta, are afflicted with xeroderma pigmentosum; they exhibit sensitivity to ultraviolet light and are prone to sunlight-induced skin cancers.

In the past year, experiments aimed at understanding the functions of Y-family polymerases spanned the evolutionary spectrum and included studies on organisms from all three kingdoms of life. In *E. coli,* the studies centered on polV and its ability to facilitate translesion replication. Scientists in the LGI previously identified, cloned, and characterized a DinB homolog from the archaeon *Sulfolobus solfataricus* P2, called DNA polymerase IV (Dpo4). In a recent collaborative study, they crystallized the enzyme and solved by X-ray crystallography the structure of ternary complexes of the polymerase together with a cyclobutane pyrimidine dimer and an incoming nucleotide. These structural studies revealed that the active site of the enzyme is sufficiently large to accommodate a covalently linked pyrimidine dimer and thus provided a model for how eukaryotic polymerases, such as human pol eta, can efficiently and accurately bypass a pyrimidine dimer.

Studies with murine DNA polymerase iota revealed that it possesses enzymatic properties similar to human pol iota in that it exhibits a remarkable template-dependent misincorporation spectrum on undamaged DNA *in vitro*. During attempts to make a targeted disruption of murine pol iota, scientists within the LGI serendipitously discovered that the commonly used 129 strain of mice carries a single nucleotide polymorphism in the *Pol iota* gene that changes the Serine 27 codon to an amber stop codon and abrogates synthesis of the polymerase. Because pol iota is an extremely error-prone polymerase, it has been hypothesized to participate in somatic hypermutation of immunoglobulin-variable genes. Analysis of somatic hypermutation in the pol iota–deficient 129 mice revealed, however, that the mice exhibit normal levels of mutagenesis and a normal mutation spectrum. Thus, it appears that either pol iota does not participate in somatic hypermutation or its role is nonessential and has been assumed by another low-fidelity DNA polymerase.

15. LABORATORY OF INTEGRATIVE AND MEDICAL BIOPHYSICS

Ralph Nossal, PhD, Chief

The Laboratory of Integrative and Medical Biophysics (LIMB) performs cross-disciplinary research to gain a deeper understanding of cell and tissue processes in both normal and disease states. It also develops new methodologies for biomedical research and diagnosis. The laboratory's work links biomedical research with experimental and theoretical techniques commonly associated with research in the physical and engineering sciences. The laboratory staff's specialized interests and expertise extend to optical imaging of biological tissues, magnetic resonance imaging, mathematical modeling, methods of quantitative cell biology, techniques for assessing ultra-small biological samples, and polymer physics and physical chemistry. The laboratory's unique capabilities include advanced physical methods such as diffuse optical tomography, magnetic resonance imaging, neutron scattering, and time-correlation spectroscopies and an ability to formulate mathematical and computational models. Investigators study biological function at levels of complexity varying from molecular to tissue, focusing on interactive behavior at different length and time scales. Much of the LIMB's work is strongly collaborative, and a number of research projects are carried out with colleagues in other NIH branches and laboratories as well as with investigators at other institutions.

Peter Basser heads the *Section on Tissue Biophysics and Biomimetics*'s, which works to gain a better understanding of tissue-level processes by developing biomimetic tissue analogs and advanced physical theories and instrumentation to aid in the design and interpretation of biological experiments. The group also continues its development of Diffusion Tensor Magnetic Resonance Imaging as a probe of tissue structure in normal or diseased organs, with particular emphasis on brain and other soft tissues.

Led by **Robert Bonner**, the *Section on Medical Biophysics* develops new optically based biophysical methodologies for research and clinical applications. The section continues its work on advancing Laser Capture Microdissection and related technologies as tools for isolating targeted cells for use in genomic and proteomic investigation of tissue pathology and in studies of developing organisms. In addition, the section is investigating the cellular basis of age-related macular degeneration, particularly as it relates to the design of optical filters to arrest disease progression.

Ralph Nossal's group, the *Section on Cell Biophysics,* aims to understand the physical basis for various cell activities that involve structural changes in supramolecular biomolecular complexes. Currently, the section's principal focus is on elucidating processes underlying the formation of vesicles involved in intracellular trafficking and on understanding the formation and transformation of supramolecular tubulin structures involved in various critical cell activities. The general long-term goal is to build and use an arsenal of tools, both theoretical and experimental, to study kinetic aspects of cell processes and to increase knowledge of ways those activities can be mediated by external interventions.

Amir Gandjbakhche's group, the *Unit on Biomedical Stochastic Physics,* works on light-tissue interactions related to noninvasive optical imaging of biological targets. The long-term objective is to develop novel approaches to tomographic imaging and quantitative optical spectroscopy of deeply buried tissue structures, including tumors. To this end, the unit is carrying out a multifaceted experimental and computational research program that incorporates mathematical and physical theories and technologies, experimental models, and collaborative clinical investigations.

16. LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT

Heiner Westphal, MD, Chief

The Laboratory of Mammalian Genes and Development (LMGD) generates gene-altered mice to study pattern formation, T cell development, and genomic imprinting. The mutant mice also serve as paradigms for human genetic disorders.

Heiner Westphal leads the *Section on Mammalian Molecular Genetics*, which carries out functional analyses of genes that control key steps of post-implantation development in the mammalian embryo. Transcription factors are major players in the cascade of molecular events that implement the body plan. The section uses a loss-of-function approach to study the function of the LIM/homeodomain (Lhx) class of transcription factors and of factors that mediate their action. Recently, the group discovered that development of cholinergic neurons in the ventral telencephalon is severely impaired in mice lacking a functional *Lhx8* gene. The loss of telencephalic cholinergic projection neurons is a hallmark of neurodegenerative disorders such as Alzheimer's disease and is likely to play a role in the cognitive impairments of such patients. For this reason, the *Lhx8* null mutant mouse constitutes a valuable model for functional studies of cholinergic projection neurons in the context of memory and cognition.

Led by **Paul Love**, the *Section on Cellular and Developmental Biology* focuses on T lymphocyte development, with special emphasis on the role of signal transduction molecules and pathways. Recent studies identified a critical role for the lymphocyte adapter protein LAT in transmitting signals from the cell surface to downstream pathways that control T cell maturation and function. These studies also demonstrated that coordination of lymphocyte signaling responses is essential for preventing inappropriate cell proliferation. In another study, researchers discovered a difference in the subunit composition of the signal-transducing antigen receptor complexes (TCRs) expressed on the two different lineages of T lymphocytes, alpha/beta and gamma/delta T cells. The data suggest that different TCR structures may influence the activation kinetics and functional responses of distinct T cell lineages. A third study, centering on the molecules that control T cell migration and trafficking, revealed a role for the chemokine receptor CCR9 in regulating T cell migration within the thymus during development.

Under the direction of **Karl Pfeifer**, the *Section on Genomic Imprinting* focuses on the regulated expression and biological function of a cluster of genes on the distal end of mouse chromosome 7. The genes share an unusual form of transcriptional regulation, genomic imprinting, that is specific to mammals. Imprinted genes are expressed from only one chromosome in a parent-of-origin–dependent manner. The section has identified a small *cis*-acting element whose epigenetic modification, established during gametogenesis, is responsible for marking the parental origin of this chromosomal region. Later, the presence of these modifications drives a developmental program that induces further changes that then cause allele-specific expression. The organization and regulated expression of the genes in this cluster are well conserved in humans, and mutations in the human genes are associated with developmental disorders, several types of tumors, and cardiac arrhythmias. The section has successfully developed a mouse model for the heart condition and has demonstrated that the cardiac deficiency is specifically attributable to the inability of the animals to respond to beta-adrenergic–mediated stress.

17. LABORATORY OF MOLECULAR GENETICS

Igor B. Dawid, PhD, Chief

The Laboratory of Molecular Genetics (LMG) studies the regulation of gene expression and the genetic control of developmental and physiological processes in viruses, bacteria, and invertebrate and vertebrate animals. Four groups in the LMG study the genetic control of development in vertebrate systems.

Led by **Tom Sargent**, the *Section on Vertebrate Development* has shown that the transcription factor AP-2 is critically required for the formation of both the epidermis and the neural crest in the frog *Xenopus laevis*.

Also working with *Xenopus*, **Igor Dawid** and colleagues in the *Section on Developmental Biology* have shown that cell movements in gastrulation that generate the embryonic axis are regulated by the transcription factor Xlim1 through its activation of the cell adhesion molecule Paraxial Protocadherin. The group has further isolated a novel feedback inhibitor of the fibroblast growth factor pathway, named Sef.

Brant Weinstein and his colleagues in the *Unit on Vertebrate Organogenesis* have made considerable progress in their study of the formation of the vascular system in the zebrafish. Using transgenic fish in which endothelial cells express green fluorescent protein (GFP), the group was able to follow cellular detail of vessel formation in the live embryo. Further, the investigators showed that phospholipase C-gamma1 is a critical factor regulating vessel formation.

Also working with zebrafish, **Ajay Chitnis**, who heads the *Unit on Vertebrate Neural Development,* and colleagues identified the gene affected in the *mindbomb* mutant as encoding a novel E3 ubiquitin ligase. The enzyme modifies the Notch ligand Delta, thereby inducing its endocytosis and enhancing Delta/Notch signaling during neurogenesis. Given the wide-ranging significance of the Notch pathway, finding a novel component in this pathway represents a major advance in the field.

Robert Weisberg and his colleagues in the *Section on Microbial Genetics* studied the mechanism of antitermination in bacteriophage lambda, a process that controls gene expression at the stage of termination of transcription.

In the area of retroviruses, **Judith Levin**'s group, the *Section on Viral Gene Regulation,* studied reverse transcription in the HIV life cycle. In particular, researchers focused on the role of nuclear capsid as a chaperone, showing that it acts to ensure effective viral DNA synthesis.

Working with bacteria, **Mike Cashel**, who leads the *Section on Molecular Regulation,* has studied the mechanism of general metabolic control that is mediated by a small molecule, guanosine tetra/pentaphosphate. He has been investigating the synthesis and degradation of this regulatory factor.

In nucleic acid metabolism, a class of enzymes that degrade RNA when duplexed with DNA plays a significant role. For some time, **Robert Crouch** has studied these enzymes, named RNasesH. In the past year, he and his colleagues in the *Section on Formation of RNA* made the interesting observation that mouse embryos lacking one form of RNaseH are not viable because they are incapable of replicating their mitochondrial (mt) DNA. The role of RNaseH in mtDNA replication is a novel and important finding that also informs hypotheses of mtDNA replication.

Using the fruit fly *Drosophila,* **Judy Kassis** and her colleagues in the *Section on Gene Expression* demonstrated the relationship between two transcriptional repressors, Pleiohomeotic and Pho-like, in the control of broad classes of genes.

For some time, **Jim Kennison** and his colleagues in the *Section on Drosophila Gene Regulation* have studied the large and important class of transcriptional activators named the Trithorax group; he discovered most of its members by genetic means. The past year witnessed the detailed analysis of the gene named *tonalli,* which interacts with other members of the group in the regulation of numerous genes.

18. LABORATORY OF MOLECULAR GROWTH REGULATION

Bruce H. Howard, MD, Chief

The Laboratory of Molecular Growth Regulation (LMGR) conducts research on the control of cell proliferation, DNA replication, and gene regulation. With respect to the last, independent groups work in complementary areas, including regulation of the developing immune system, gene expression during early embryogenesis, chromatin-mediated gene silencing, and transcription of small RNA–encoding genes.

Of interest in the immune system are transcription factors that control cell proliferation and differentiation. Studies by **Keiko Ozato**'s group, the *Section on Molecular Genetics of Immunity,* have revealed that ICSBP/IRF-8 is important for the proper development of macrophages, granulocytes, and dendritic cells. Defects in these cell lineages can be rescued by introduction of the ICSBP gene into cells derived from knock-out mice. Both ICSBP and Brd4, another transcription factor under investigation, have been shown to function by regulating the activity of chromatin. Brd4 further interacts via its bromodomain with the DNA replication factor RFC.

A separate area of investigation, control of gene expression during early mouse embryogenesis, has been linked to the transcription factor mTEAD-2. Experiments conducted by the *Section on Eukaryotic Gene Regulation*, led by **Mel DePamphilis**, have identified YAP65 as a long sought-after coactivator of the TEAD family of transcription factors. YAP65 is a strong candidate for transducing mitogenic signals to TEAD-dependent transcription. An independent project concerns cell-cycle control and the DNA replication factor ORC1. ORC1 was shown to be ubiquitinated and released from chromatin during S phase. Subsequent ORC1 binding to chromatin is blocked by Cdk1/cyclin A.

Still another area of study relates to the multifunctional factor known as human La antigen. Recently, La antigen has been characterized as a regulatory chaperone for nascent RNAs. Work in the *Section on Molecular and Cell Biology,* under the direction of **Richard Maraia**, has been instrumental in defining the roles of La in the nuclear trafficking and successful processing of tRNAs. Related studies have highlighted the regulatory function of a major phosphorylation site in La antigen. In this case, phosphorylation modulates the interaction of La with nascent transcripts as well as its localization to the nucleolus.

A major effort of the *Human Genetics Section*, led by **Bruce Howard**, focuses on higher-order chromatin structure, in particular how defects in the maintenance of such structures may underlie developmental disorders and age-related diseases. The laboratory has developed random genome sampling, custom search algorithms for genome comparisons, and genomics-style high-throughput approaches to facilitate the mapping of chromatin structures as well as to permit the detection of areas of chromatin remodeling.

A new research group led by **David Clark**, the *Section on Chromatin and Gene Expression,* has joined the laboratory and brings expertise in both yeast genetics and the characterization of purified chromatin structures.

19. LABORATORY OF PHYSICAL AND STRUCTURAL BIOLOGY

V. Adrian Parsegian, PhD, Chief

The research conducted by the Laboratory of Physical and Structural Biology (LPSB) is motivated by the need to bring together many types of science. The next step in structural biology is not simply to determine the structure of every identifiable entity from molecule to organelle. Rather, it is to learn how the structures work through the physics and chemistry of the intermolecular forces that create them. Then, it will be possible to learn from the increasing number of protein, nucleic acid, saccharide, and lipid structures how to design agents that compete effectively with deviant interactions associated with disease.

Based on observations of penicillins moving through protein channels, members of the *Section on Molecular Transport,* led by **Sergey Bezrukov**, have shown how attraction between channel and penetrating molecules improves the probability of successful molecular traverse. Instead of drifting back out the side it entered, an antibiotic that is attracted to the channel interior rattles about long enough to forget which way it came in. A 2 percent likelihood of full traverse becomes a 50 percent chance of coming out the other side, at only a small cost in the time spent in the channel.

Osmotic stress measurements of specific versus nonspecific associations of proteins with DNA have shown that sequences differing from the specific sequence by even a single base pair bind only slightly better than completely random sequences. This abrupt decrease in binding energy with even a single base pair change is accompanied by an abrupt increase in the water sequestered by the protein-DNA complex. These stress studies, conducted by **Donald Rau**'s group, the *Unit on Macromolecular Recognition and Assembly,* reveal the essential role of dehydration in the tight fit needed for sequence recognition as well as the importance of the ability to glide along the solvating water of nonspecific associations until the protein finds its spot.

Combining computer computation with theoretical methods of solution chemistry has allowed **Adrian Parsegian**'s group, the *Section on Molecular Biophysics,* to develop a new simulation method for swift modeling of protein folding under test-tube and cellular solution conditions rather than relying on the artificial conditions of the usual simulation box. In parallel with the use of physical theory for efficient computation, the section is measuring responses in molecular association and the organization of lipids that are wrought by changes in the chemical potential of neutral solutes and salts.

20. PEDIATRIC AND REPRODUCTIVE ENDOCRINOLOGY BRANCH

George P. Chrousos, MD, Chief

The Pediatric and Reproductive Endocrinology Branch (PREB) had a long list of published research achievements in the areas of glucocorticoid action, interactions between the HPA axis and immune and inflammatory reactions, and preclinical studies with the CRH receptor type 1 antagonist antalarmin, congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency, hypercortisolism, endometriosis, anovulation, infertility, pheochromocytoma, uterine fibromas, and the novel *Brx* gene. Some of the branch's achievements are highlighted below.

The *Section on Pediatric Endocrinology*, led by **George Chrousos** elucidated the cellular pathophysiology of two new probands with familial/sporadic glucocorticoid resistance, describing three novel mutations of the glucocorticoid receptor and abnormalities in nuclear translocation and interactions with p160 coactivators. Members of the section demonstrated both inadequate cortisol secretion and pro-inflammatory cytokine–induced, NF-kappaB–mediated glucocorticoid resistance in patients with acute respiratory distress syndrome/systemic inflammation. They also demonstrated that FLASH, a plasma membrane–associated protein that mediates the proapoptotic effects of tumor necrosis factor alpha and Fas Ligand, is involved in inflammation-associated glucocorticoid resistance. They described two HIV-1 small accessory proteins, Vpr and Tat, as viral glucocorticoid receptor coactivators, proteins that were shown to participate in a similar coactivator complex in the HIV-1 LTR promoter. They demonstrated Vpr-mediated glucocorticoid hypersensitivity in several different systems, including interleukin-12 suppression, and discovered a dominant-negative Vpr mutant that can eliminate the coactivator activity of Vpr. The section established that a single oral dose of antalarmin inhibits stress behaviors in rhesus monkeys. Investigators found that, in rats, antalarmin ameliorates adjuvant-induced arthritis, blocks ulcer formation in stressed animals, and, by inhibiting aseptic inflammation and expression of Fas-ligand by the invasive cytotrophoblast, blocks blastocyst implantation. Studies on CAH established that an antiandrogen and an aromatase inhibitor control bone age and growth and allow smaller doses of glucocorticoids in children with severe classic CAH, that the disease is associated with severe adrenomedullary dysfunction, which correlates with the degree of the enzymatic defect and the molecular defect, that the epinephrine deficiency of CAH is associated with an inability of patients to increase their circulating glucose levels during exercise, that CAH is associated with insulin resistance and increases in plasma leptin level, and that children with CAH have structural changes in the brain, with significantly diminished amygdala size.

Under **Lynette Nieman**, the *Section on Reproductive Medicine* demonstrated marked comorbidity of endometriosis, autoimmune disorders, and fibromyalgia. Nieman also established that midnight plasma cortisol or salivary cortisol is an excellent diagnostic test to distinguish Cushing's syndrome from pseudocushing states and demonstrated the utility of SPGR MRI for the identification of corticotrope tumors and of etomidate and octreotide for the treatment of Cushing's syndrome.

Led by **Karel Pacak**, the *Unit on Clinical Neuroendocrinology* found that plasma-free metanephrine levels are the best biochemical test for the diagnosis of pheochromocytoma, either alone or in combination with the clonidine suppression test. The unit also developed pediatric reference ranges for plasma-free metanephrines and validated their use for the diagnosis of childhood pheochomocytoma. Investigators developed the 6-[^{18}F]-fluorodopamine PET scan for the diagnostic localization and follow-up of pheochromocytoma and the novel use of radiofrequency ablation in the treatment of pheochromocytoma.

The *Unit on Reproductive Endocrinology and Infertility* investigated a variety of clinical reproductive disorders. Studies that were an integral component of the Reproductive Endocrinology and Infertility Training Program, directed by **James Segars**, led to identification of the genes that contribute to uterine leiomyoma tumorigenesis as well as demonstration of the major role of Brx in the estrogen receptor and protein kinase signaling systems.

21. PERINATOLOGY RESEARCH BRANCH

Roberto Romero, MD, Chief

The Perinatology Research Branch (PRB) conducts clinical and laboratory research in maternal and fetal diseases responsible for excessive infant mortality in the United States. The PRB focuses on the mechanisms of disease underlying premature labor and delivery, with particular emphasis on the role of subclinical intrauterine infection. The prenatal diagnosis of congenital anomalies is also a major area of interest.

NICHD awarded a major contract and lease to establish a facility and support the operations of the PRB in the state of Michigan. The new campus will offer access to a patient population with a high rate of pregnancy complications and brings to bear the academic resources of three major universities: Wayne State University, University of Michigan, and Michigan State University. The PRB plans to develop strong collaborative relationships with these universities and the Van Andel Research Institute. These organizations are part of the Life Science Corridor established by the state of Michigan to attract and support research and development in the biomedical field. The clinical and laboratory facilities of the PRB will open on July 1, 2004. The development of the physical plan, organization, and recruitment of support staff will continue to be a major focus of the branch's efforts.

Premature birth is the leading cause of perinatal mortality and morbidity worldwide. The PRB has defined preterm labor as a syndrome and determined that at least 25 percent of all preterm infants are born to women with sub-clinical intrauterine infection. Moreover, the branch has provided evidence that a substantial number of premature neonates are critically ill before birth; thus, it has proposed that the onset of premature labor has survival value in the context of intrauterine infection. This year, the PRB conducted several studies to explore the role antibiotic administration in the treatment of intrauterine infection and also the value of assessing the concentration of antimicrobial proteins in the detection of intrauterine infection and inflammation.

After premature birth, congenital anomalies are the second leading cause of perinatal mortality in the United States. Advances in imaging techniques have permitted the use of ultrasound before birth to detect many anatomical defects. The PRB is currently using three- and four-dimensional ultrasound to delineate normal fetal anatomy and improve the detection of congenital anomalies and estimation of fetal weight and growth. This year, PRB has used four-dimensional ultrasonography with spatio-temporal image correlation for the evaluation of the fetal heart and three-dimensional ultrasound to quantitate fetal lung volume measurements.

22. PHYSICAL DISABILITIES BRANCH

Steven J. Stanhope, PhD, Chief

The Physical Disabilities Branch (PDB) was established in 2001 in part to accelerate the development of a highly collaborative, multidisciplinary, technology development and rehabilitation research network. To lend focus to the network, the branch adopted a central programmatic research theme that stresses the decompartmentalization of rehabilitation research efforts.

To develop advanced technologies for basic, translational, and clinical rehabilitation research, scientists and engineers working within the *Biomechanics and Biomedical Engineering Section* developed a movement analysis methodology structured on a generalized coordinate-based mechanical linkage model that can provide direct estimates of the influence of a given joint moment or muscle force on the movement of all joints and body segments as well as on overall task performance. In addition, PDB staff and affiliates, who previously demonstrated the feasibility of studying musculoskeletal kinematics with cine-Phase Contrast (PC) and fast-PC magnetic resonance imaging techniques, are working to define static and dynamic measures of patellar tracking abnormalities as a potential source of patellofemoral pain.

The mission of the *Human Movement Disorders Section* is to understand human function and the influence of rehabilitation interventions in the context of activities of daily living in order to optimize function for people with physical disabilities across the lifespan. Recent accomplishments include a testing procedure to determine the mechanical properties of orthoses, the development of models to allow quantification of an orthotic's contribution to ambulation, and evaluation of the effect of an orthotic on lower extremity function during gait. Studies on the governing principles linking impairment to functional movement limitations in the elderly have demonstrated a hierarchy of compensatory movement strategies whereby maintaining standing ability is strongly influenced by the functional status of the subject and the difficulty of the movement task required.

The *Oral Motor Function Section* strives to understand normative and adaptive oral motor functions. Researchers recently documented differences in the growth and maturation of the upper aerodigestive system between the normal and at-risk fetus; lingual perfusion and reperfusion patterns as a function of physiological task performance; swelling in the oral cavity after removal of the third molars, as determined by ultrasound imaging; progression of swallowing and oral motor function in nephropathic cystinosis; anatomic and behavioral components of the fetal upper aerodigestive system from 15 to 39 weeks gestation in normal and abnormal births; the use of MRI lingual volumetrics for tongue modeling; and temporal coordination and swallow-related response characteristics of the suprahyoid and infrahyoid musculature. They also conducted pilot studies to test the use of tagged and diffusion tensor MRI to study internal lingual mechanical function and muscle architecture.

23. SECTION ON PHYSICAL BIOCHEMISTRY

Sergey Leikin, PhD, Chief

Physicists and chemists who staff the Section on Physical Biochemistry (SPB) conduct experimental and theoretical studies on the structure and function of biomolecules with an emphasis on molecular mechanisms of pathology in connective tissue disorders. Through collaboration with clinical researchers, they develop novel techniques for diagnostics, characterization, and treatment of osteogenesis imperfecta (OI) and other diseases.

Section scientists reported the first direct measurements and established the physical nature of forces between collagen molecules. They discovered that the collagen triple-helix is intrinsically unstable under physiological conditions, that micro-unfolding of most thermally labile helical regions is necessary for proper molecular recognition, and that, apparently, endoplasmic reticulum chaperons "forcibly" fold collagen precursor (procollagen). Currently, the section is gradually shifting from fundamental studies of this process to understanding how different OI mutations affect it. For instance, in collaboration with members of the Bone and Extracellular Matrix Branch, section researchers recently revealed an important folding domain formed by the first 90 amino acids of the collagen triple helix. They discovered that OI mutations within this region disrupt the structure of the whole domain and cause abnormal cleavage of N-propeptides and that incorporation of uncleaved molecules into fibers produces smaller diameter fibers with reduced strength, causing early onset scoliosis, hyperextensibility, and joint laxity in patients.

Section scientists uncovered several common physical principles governing the formation, structure, and physical properties of collagen and DNA aggregates. They suggested mechanisms for counter-ion specificity in DNA condensation, for DNA overwinding from 10.5 base pairs per helical turn in solution to 10.0 bp per turn in aggregates, and for sequence homology recognition in pairing of duplex DNA. Investigations currently focus on measurement of predicted sequence effects in formation, structure, and properties of DNA aggregates.

24. SECTION ON NERVOUS SYSTEM DEVELOPMENT AND PLASTICITY

R. Douglas Fields, PhD, Chief

Research in the Section on Nervous System Development and Plasticity (NSDP) is concerned with the cellular and molecular mechanisms of nervous system development, with particular emphasis on how neural impulse activity regulates development and plasticity of the nervous system. A molecular and electrophysiological approach is combined with live cell confocal imaging to investigate how patterned neural impulse activity is transduced into intracellular signaling networks to regulate expression of specific genes controlling development and synaptic plasticity. The scope of the section's investigations extends to activity-dependent interactions between neurons and glia and synaptic plasticity in the hippocampus. In cell culture and acute brain slice, the section studies mechanisms of activity-dependent communication between neurons and glia and the intracellular signaling pathways controlling gene expression necessary for long-term memory. The section's research has shown effects of impulse activity on cellular differentiation, axon outgrowth, synaptogenesis, synaptic remodeling, and myelination.

25. SECTION OF CELL BIOLOGY AND SIGNAL TRANSDUCTION

James T. Russell, DVM, Chief

The Section on Cell Biology and Signal Transduction focused almost exclusively on characterization of Ca^{2+} signaling microdomains at the structural and functional levels. Signaling microdomains are specialized cellular Ca^{2+} release sites that produce a regenerative Ca^{2+} release at specific cellular loci such that they support propagation of calcium waves through a cell and its processes. With confocal microscopy, the section carried out a comprehensive analysis of the kinetics of Ca^{2+} release from these specialized release sites and identified sites of Ca^{2+} sparks and Ca^{2+} puffs in oligodendrocyte progenitor cell processes. These sites of Ca^{2+} release corresponded to cellular locations where IP_3Rs are expressed in clusters. In addition, a number of other proteins involved in Ca^{2+} homeostasis and Ca^{2+}-based signaling are found clustered at these specialized sites. The findings lead to the hypothesis that microdomains of Ca^{2+} release exist in cells to achieve locally discrete Ca^{2+} signals while supporting regenerative wave propagation. Currently, a comprehensive characterization of the proteins that cluster in the microdomains is under way. In a related study, the section is characterizing the signaling complex organized at the nodes of Ranvier in myelinated axons.

WORKSHOPS AND SYMPOSIA ORGANIZED BY NICHD INTRAMURAL RESEARCH PROGRAM STAFF IN 2002-2003

International workshop "Protein trafficking: molecular mechanisms and disease"
This four-day workshop, held in October, 2003, in Pucon, Chile, was organized by Juan Bonifacino, CBMB, together with Enrique-Rodriguez Boulan, Cornell Medical School, and Alfonso Gonzalez, Center for Cell Regulation and Pathology (CCRP), Chile. It was cosponsored by the NICHD, the American Society for Cell Biology, CCRP, and the Millenium Institute for Fundamental and Applied Biology, Chile. The workshop served as a forum for the presentation and discussion of recent findings on the role of protein trafficking pathways in the biogenesis of endocytic and secretory organelles and on the diseases resulting from dysfunction of these pathways. Several sessions examined the molecular machineries that sort proteins to different the Golgi complex, endosomes, lysosomes, and the plasma membrane. Specific topics discussed included sorting signals and their cognate adaptors, the generation of transport vesicles, and the participation of cytoskeletal motors in the movement of vesicular and tubular transport carriers. Important physiological processes analyzed in relation to these molecular mechanisms included the regionalization of specific domains of the plasma membrane, epithelial morphogenesis, the biogenesis of lysosomes and synaptic function, in each case considering examples of altered processes that lead to disease. The following prominent scientists in the field participated: Willem Annaert, Universiteit Leuven, Belgium; Alfredo Cáceres, Instituto Mercedes y Martín Ferreyra, Universidad de Córdoba, Argentina; Pietro de Camilli, School of Medicine, Yale University; Victor Faúndez, Emory University School of Medicine; John Hammer, NIH; Kathryn Howell, School of Medicine, University of Colorado; Vivek Malhotra, University of California; Keith Mostov, University of California, San Francisco; Sandra Schmid, The Scripps Research Institute; Thomas Söllner, Sloan Kettering Institute.

Inverse Problems and Medical Imaging
This four-day workshop, held in Vancouver on August 2003, was organized by the prestigious Pacific Institute for the Mathematical Sciences of University of British Columbia, and supported by the NSF. Amir H. Gandjbakhche, LIMB, was among the organizers of this event. The workshop focused on recent developments in medical imaging, particularly the advances in mathematics that have allowed significant enhancement of widely used imaging techniques such as x-ray tomography, magnetic resonance imaging, and ultrasonic imaging. Mathematical developments in emerging medical imaging modalities, such as optical imaging, were also surveyed. The aim of the workshop was to bring together investigators working on different aspects of these fields and to encourage interaction between mathematicians, physicists, and physicians. Thirty international experts including Dr. Gandjbakhche, were among the invited speakers, who identified critical limits and prospects of mathematical inverse problems in different imaging modalities.

The Genetic Code Revisited: the Impact of Functional Genomics in Medical Research
This symposium on functional genomics, in honor of Marshall W. Nirenberg, was held on December 16, 2002, at the Natcher Conference Center at the NIH. Marshall Nirenberg was awarded the Nobel Prize in Medicine in 1968 for his ground-breaking, landmark studies in the 1960s that culminated in the elucidation of the genetic code. The studies laid the intellectual foundation for the extraordinary progress in biology that we have witnessed over the last 35 to 40 years and led to the new era of functional genomics, which will have a critical impact on molecular medicine, understanding of human disease, and, ultimately, improvement of human health. The day-long symposium consisted of three sessions: "The Genetic Code and the Biology of the Cell"; "Strategic Uses of Functional Genomics"; and "Therapeutic Advances through Genomics." The speakers were prominent scientists who work in the genomics field, including researchers trained in Dr. Nirenberg's laboratory.

Welcoming remarks were delivered by Elias Zerhouni, NIH director. Over 800 people attended the event, which was organized by three former postdoctoral fellows who were in Dr. Nirenberg's group in the 1960s and are still working at the NIH: Dolph Hatfield, chief, Section on the Molecular Biology of Selenium, Basic Research Laboratory, NCI; Samuel Wilson, deputy director, NIEHS; and Judith G. Levin, chief, Section on Viral Gene Regulation, LMG, NICHD. Dr. Nirenberg's institute at the NIH, the National Heart, Lung, and Blood Institute, sponsored the event. A detailed description of the symposium was published in the *NIH Record* of January 21, 2003 (vol. LV, no. 2).

Fourth International Retroviral NC Symposium

Judith Levin also served on the Scientific Committee for the of this three-day symposium, held in September, 2003, in Strasbourg, France. The retroviral nucleocapsid protein (NC) is a small, highly basic, nucleic acid-binding protein, which has one or two zinc fingers, each containing the invariant CCHC motif. It is now known that NC functions in multiple steps in virus replication. In view of the critical importance of this protein in the life cycle of the retroviruses and especially HIV, a symposium is organized every two years for researchers in this field. This year's conference featured sessions on the interaction of NC with nucleic acids with reports on structural and physicochemical studies and the role of NC in reverse transcription, recombination, integration, and encapsidation of genomic RNA. Other discussions focused on the role of NC and other retroviral proteins in virus assembly and new developments in NC-based HIV vaccines and anti–NC drugs.

World Congress on Iron Metabolism

The 16th International Conference of the International BioIron Society, held concurrently with the 6th International Symposium on Microbial Iron Transport, Storage and Metabolism in May, 2003, at the NIH was organized by Tracey Rouault, chief, Section on Human Iron Metabolism, CBMB, and Victor Gordeuk, Professor of Medicine, Center for Sickle Cell Disease and Department of Medicine, Howard University. During the five-day meeting, sessions were devoted to such topics as regulation of iron metabolism, hemochromatosis, iron and neurodegenerative diseases, iron and the response to hypoxia, mitochondrial iron metabolism and iron sulfur cluster biogenesis, hepatic iron metabolism and treatment of hepatitis, and iron chelation and iron overload in sickle cell disease, thalassemia, and other iron-loading anemias.

Laser Capture Microdissection and Macromolecular Analysis of Normal Development and Pathology

Robert Bonner has continued to organize and hold international conferences at NIH on Laser Capture Microdissection (LCM) and Macromolecular Analysis of Normal Development and Pathology, this year bringing together about 500 researchers to discuss research and methodological advances made possible by LCM.

International Conference on Delayed and Nondisclosure of Child Sexual Abuse

In August 2003, Michael Lamb and his staff in the Section on Social and Emotional Development and their collaborators convened an international conference on delayed and nondisclosure of child sexual abuse; a book based on the conference proceedings is in preparation.

Pineal Microarray Workshop

David Klein and his colleagues organized and sponsored of the first Pineal Microarray Workshop. The workshop was held at the Airlie Conference Center, Coolfont, WV, in January, 2003, to provide investigators from six laboratories an opportunity to present and discuss their results from microarray-based gene expression analysis of the pineal gland. The work presented included studies using three experimental platforms, and tissues from three mammals and one avian species. The workshop was successful in accelerating the exchange and assessment of the results of this body of work, much of it unpublished at the time the meeting was held. A major outcome was the identification of sets of genes that are highly expressed in the pineal gland of all the species examined, establishing those that are common pineal genes. In addition, identification of a number of genes that are highly expressed in only one species suggests that the pineal may also have pineal-specific functions.

RESEARCH PROJECTS

OF THE

LABORATORIES

AND

BRANCHES

CONNECTIVE TISSUE DISORDERS*

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Emily Moriarty, AB, *Postbaccalaureate Fellow*

In a unique integrated program of laboratory and clinical investigation, we study the molecular biology of the heritable connective tissue disorders osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome (EDS). Our objective is to elucidate the mechanisms by which primary collagen defects cause skeletal fragility and other significant connective tissue symptoms and then to apply the knowledge gained from our studies to the treatment of children with these conditions. An understanding of the interactions of mutant collagen molecules with the normal collagenous and noncollagenous components of extracellular matrix will also enhance our understanding of normal bone function and may yield insights applicable to the more common forms of osteoporosis. We recently focused on the development of a nonlethal animal model for OI with a classical collagen mutation. The nonlethal knock-in mouse, the Brtl mouse (Brtl), with a glycine substitution mutation in the alpha1(I) chain, is an excellent model for pharmacological treatment trials, for approaches to gene therapy suitable for dominant disorders, and for investigations of the skeletal matrix of OI. Our clinical studies involve children with types III and IV OI who are enrolled in age-appropriate clinical protocols for treatment and who form a longitudinal study group.

The OI/EDS region of the alpha1(I) collagen chain

Cabral, Letocha, Marini; in collaboration with Leikin

Patients with OI/EDS form a distinct subset of OI patients. In addition to the skeletal fragility of OI, they have characteristics of EDS, including severe laxity of large and small joints and early-onset scoliosis. In seven children with OI/EDS, we delineated mutations in the first 90 residues of the helical region of the alpha1(I) chain. We have determined that these collagen mutations cause abnormal N-propeptide processing, incorporation of pN-collagen into matrix, and decreased diameter of dermal fibrils. The data point to a mechanism for the children's EDS symptoms while the helical changes *per se* are responsible for bone fragility. Thus, the mechanism of their EDS is shared with patients who have EDS VIIA and B due to absence of the N-proteinase cleavage site from the alpha1(I) or alpha2(I) chain, respectively.

Given that the proband collagen was electrophoretically normal, we identified mutations by direct sequencing of RT-PCR amplification products of alpha1(I) mRNA. Mutations in exon 7 resulted in helices with both decreased and normal T_m (melting temperature) visible as a double peak on differential scanning calorimetry (DSC) of proband collagen and as a broadened single peak on DSC of proband procollagen. Mutations in exons 8 to 11 showed even lower T_m of mutant helices with

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1. Bone and Extracellular Matrix Branch

well-separated mutant and normal DSC peaks in collagen and overlapping, but still distinct, DSC peaks in procollagen. All seven mutants had different thermal stability of collagen and procollagen, in sharp contrast to mutants beyond the first 90 amino acids whose procollagen and collagen DSC traces were identical. *In vitro* cleavage with N-proteinase processed only 25 percent of proband proalpha1(I) chains for exon 7 mutations and 65 to 85 percent of proalpha1(I) chains for exon 8 to 11 mutations. The pericelluar processing of the seven mutants was also delayed. The pN-collagen is incorporated into matrix deposited by cultured fibroblasts, with pN-alpha1(I) collagen prominently present in the newly incorporated and immaturely cross-linked fractions. Using electron microscopy to examine the dermal fibrils of six patients, we observed that all six patients' fibril diameters were significantly smaller than those of matched controls, as is seen in EDS VII. The fibril diameter was smallest for mutations in exons 7 and 8 and intermediate in size for mutations in exon 11.

The assays define a folding region of alpha1(I) in which mutations cause a distinct OI/ED phenotype by altering the triple helical structure and secondary structure of the N-proteinase cleavage site. The retention of N-propeptide in a substantial proportion of collagen chains limits fibril diameter. The abnormal fibrils may cause laxity of joints and paraspinal ligaments directly by reduced resistance to shearing forces or indirectly by altering interactions between collagen and other matrix components in the overlap zone of the D-periods.

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Alendronate treatment of Brtl mouse

Marini, Uveges; in collaboration with Goldstein, Gronowicz

Bisphosphonate drugs are widely administered to children with OI, but their effects on OI bone tissue containing abnormal type I collagen have not been directly examined. The Brtl mouse model for type IV OI has a glycine substitution (G349C) knocked into one COL1A1 allele. We treated Brtl and wild-type offspring of Brtl x CD-1 matings from two to 14 weeks of age with alendronate (0.219 mg/kg/wk, gift of Merck) or saline placebo. Brtl mouse weight and femoral length were significantly smaller than wild type and unchanged by alendronate.

Whole-bone density of femurs and lumbar vertebrae, measured with a Lunar Piximus, were significantly increased in both treated Brtl and wild type; treated Brtl samples attained average untreated wild-type bone mineral density (BMD). Micro CT data suggest that the differences in Brtl are attributable to increases in bone volume rather than to mineralization. Distal femoral bone volume per total volume doubled with treatment in both Brtl and wild type as a consequence of increased trabecular number. Diaphyseal cortical thickness increased by periosteal bone deposition in Brtl and wild-type femurs. In treated Brtl femurs, overall geometry was reshaped to a more rounded structure.

We tested the mechanical properties of femurs in four-point bending. Alendronate treatment increased yield load, ultimate load, and stiffness in both Brtl and wild type. However, alendronate failed to improve the brittleness of the Brtl femoral bone; postyield displacement remained significantly lower in treated Brtl than in wild type. Increased cortical shattering during longitudinal sectioning supports the conclusion of greater brittleness of treated Brtl bones.

Osteoclast surface was increased by 60 percent in both wild-type and Brtl femurs. Osteoblast surface increased significantly in wild type but was unchanged in Brtl. In alendronate-treated Brtl, fewer plump cuboidal osteoblasts were seen; many osteoblasts had an intermediate morphology with enlarged Golgi, suggesting functionally exhausted cells. Our interpretation is that alendronate treatment of Brtl increases the amount of new periosteal and trabecular bone, leaving synthetically depleted osteoblasts at 14 weeks. Because of the increased posttreatment bone volume, Brtl femurs can withstand a larger load before undergoing permanent deformation. However, femur brittleness is not improved and may be exacerbated. After reaching the yield point, Brtl femurs will fracture with similar additional load and deformation as if untreated.

Pamidronate treatment of children with types III and IV OI

Letocha, Marini; in collaboration with Gerber, Paul

Uncontrolled trials of bisphosphonates in OI children report increased vertebral bone density and height, improved strength and functional level, and decreased fractures and bone pain. We undertook a controlled trial of pamidronate in children with Types III and IV OI. Children in the treatment group received pamidronate (10 mg/m^2/day for three days every three months); all children had quarterly rehabilitation and physical therapy assessments, including measurements of function, strength, and pain. Treated patients experienced a significant increase in vertebral BMD z-score compared with controls. They also had significant increases in L2 vertebral height and L1 and L2 area.

In contrast to reports from uncontrolled trials, we found no significant changes in ambulation level, lower extremity strength, or pain in OI children treated with pamidronate. We assessed motor skills related to ambulation with the 10-point Brief Assessment of Motor Function (BAMF). The treatment group BAMF at initiation was 6.1±1.8 versus 6.7±1.9 at 12 months ($p = 0.5$). The control group BAMF at initiation was 6.6±2 versus 7.02±1.31 at 12 months ($p = 0.61$). Both groups, on average, used maximum gait aids. Manual muscle testing was assessed as the sum (total points 110) of abdominal, straight leg raise, hip abduction, extension, and flexion, and quadricep strength. Lower-extremity muscle strength did not change. The treatment group score was 67.1±20.9 at baseline versus 68.6 ± 19.7 at 12 months ($p = 0.88$); the control group score was 74.2±21.3 at initiation versus 74.6±14.3 at 12 months ($p = 0.97$). There was no significant decrease in pain on a four-point scale. Some patients reported increased endurance or decreased back pain, but most patients reported no perceptible changes.

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Until October, 2003, this research was conducted in the Section on Connective Tissue Disorders of the Heritable Disorders Branch.

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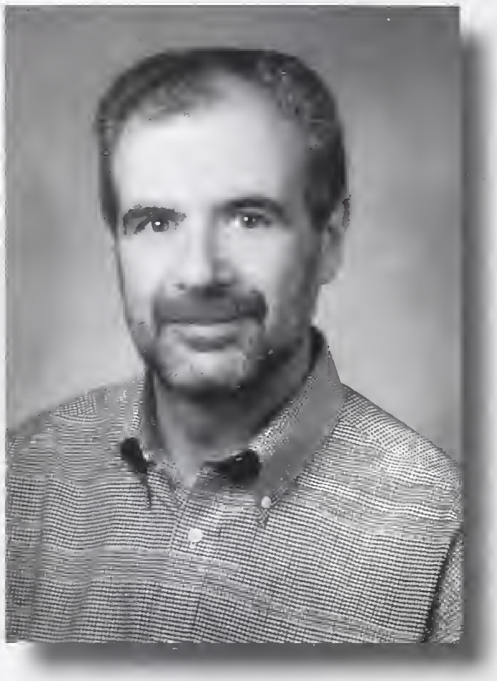
PROTEIN SORTING IN THE ENDOSOMAL-LYSOSOMAL SYSTEM



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We investigate the molecular mechanisms that control the sorting of transmembrane proteins in the endosomal-lysosomal system. Sorting processes such as rapid internalization of receptors from the plasma membrane, targeting to lysosomes and lysosome-related organelles, and delivery to the basolateral plasma membrane domain of polarized epithelial cells are all mediated by interactions between signals in the cytosolic domains of the transmembrane proteins and adaptor proteins associated with the cytosolic face of membranes. Previous research has described two major types of sorting signals: tyrosine-based and dileucine-based signals. Our work as well as that of other laboratories has demonstrated that both signal types are recognized with characteristic fine specificities by the adaptor protein (AP) complexes AP-1, AP-2, AP-3, and AP-4 or by the GGA adaptor proteins GGA1, GGA2, and GGA3 (see Figure 2.1). Mutations in AP-3 are the cause of the pigmentation and bleeding disorder Hermansky-Pudlak syndrome type 2. Our current work focuses on elucidating the structure, regulation, and physiological roles of the AP complexes and GGAs and on investigating the possibility that defects in these proteins underlie protein trafficking disorders.

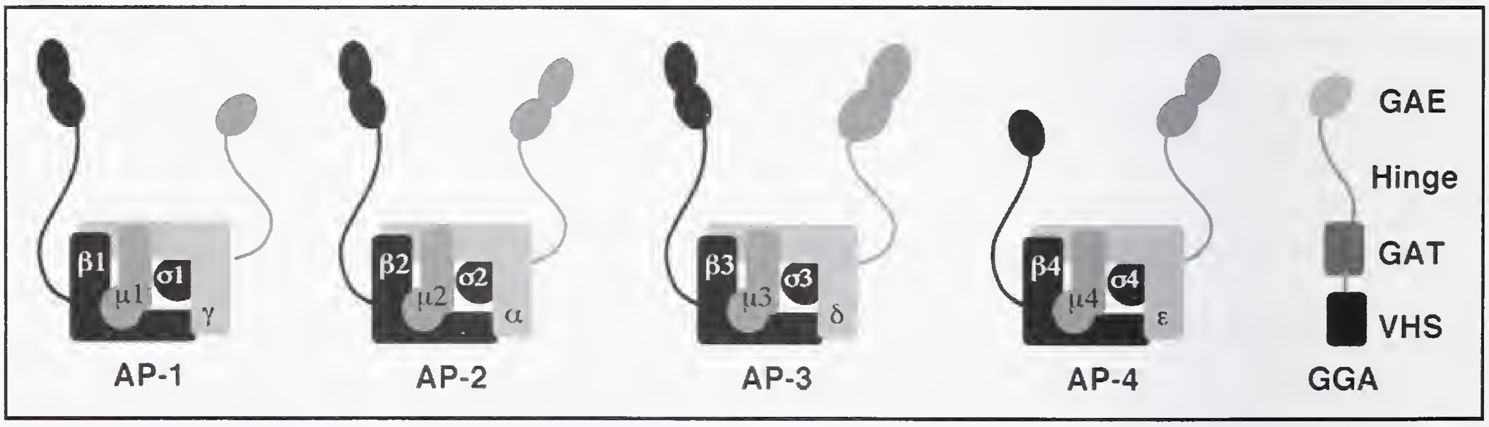

FIGURE 2.1

Structure of AP complexes and GGAs.

Role of the GGA adaptor proteins in the biogenesis of lysosomes

Mattera, Puertollano, Bonifacino; in collaboration with Hurley, McPherson, Miller, Ritter

Over the past year, we have continued our studies on the structure and function of the GGA proteins. The GGAs are modular proteins composed of four domains named VHS, GAT, hinge, and GAE. Each domain has a different function: the VHS domain is a recognition module for dileucine-based sorting signals present in the cytosolic domains of the mannose 6-phosphate receptors (MPRs) that sort acidic hydrolases to lysosomes; the GAT domain binds to Arf-GTP and targets the GGAs to the trans-Golgi network (TGN); the hinge domain recruits clathrin; and the GAE domain binds to accessory factors involved in vesicle budding and fusion and in interactions with the cytoskeleton. These properties indicate that the GGAs' function as Arf-dependent adaptors for the recruitment of clathrin to the TGN. In addition, they participate in the sorting of MPRs and their cargo, the lysosomal hydrolases from the TGN. This past year, we identified a canonical peptide motif that mediates the interaction of accessory proteins with the GAE domain of the GGAs and the related "ear" domains of the gamma-adaptin subunit of AP-1. The specification of this motif has allowed us to identify novel accessory proteins involved in protein sorting at the TGN. In collaboration with James Hurley's group (NIDDK), we solved the crystal structure of the GAE domain of human GGA3 in complex with a Phe-Gly-Pro-Leu-Val peptide, which was derived from the accessory protein Rabaptin-5 and which conforms to the canonical peptide motif. The structure revealed that the Phe and Leu of the peptide bind to hydrophobic pockets formed by the aliphatic portions of basic residues on the GAE domain. The Gly and Pro residues allow the peptide to bend such that the hydrophobic residues can be properly aligned.

The properties of the GGAs described above are indicative of a role in the sorting of acidic hydrolases to lysosomes. However, the evidence for such a role is indirect. To investigate the physiological roles of the human GGAs, we have taken advantage of the ability to suppress expression of mammalian proteins with small interfering RNAs (siRNAs). With this methodology, we have reduced by 80 to 90 percent the cellular levels of GGA1, GGA2, GGA3, and the AP-1 complex. Depletion of GGA3 as well as combinations of GGA1 and GGA2 with AP-1 caused mislocalization of the MPRs to enlarged early endosomes. These manipulations also resulted in secretion of the unprocessed precursor of the lysosomal hydrolase cathepsin D into the medium. Over a period of a week of siRNA treatment, lysosomes became enlarged probably in response to an accumulation of materials that could not be degraded because of the mis-sorting of lysosomal hydrolases. This phenotype was reminiscent of I-cell disease, in which a defect in lysosomal hydrolase sorting gives rise to giant lysosomes. These experiments have thus provided the first direct line of evidence that the GGAs are involved in the biogenesis of lysosomes. Experiments are now under way to determine whether defects in the GGAs underlie any lysosomal storage disorders.

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Molecular mechanisms of protein sorting

Janvier, Boehm, Martina, Caplan, Kim, Kato, Bonifacino; in collaboration with Backlund, Venkatesan*

In addition to the GGAs, other proteins play important roles in protein sorting in the endosomal-lysosomal system. Among these proteins are the AP complexes AP-1, AP-2, AP-3, and AP-4, which are composed of four subunits and are part of protein coats associated with the cytosolic face of membranes at sites of protein sorting. The AP complexes recognize a subset of dileucine-based sorting signals distinct from those recognized by the GGAs. While the signals play important roles in endocytosis and lysosomal targeting, the identity of the AP subunits that harbor the binding sites for the dileucine-based sorting signals remained to be elucidated. We used a yeast three-hybrid assay to demonstrate that the dileucine-based sorting signals from the human immunodeficiency virus *Nef* gene product and the lysosomal membrane protein LIMP II interact in a bipartite manner with combinations of the gamma and sigma1 subunits of AP-1 and with the delta and sigma3 subunits of AP-3, but not with the analogous subunits of AP-2 and AP-4. The sequence requirements for interactions in these assays are similar to those for binding to the whole AP complexes *in vitro* and for the function of the signals *in vivo*. Our observations have revealed a novel mode of recognition of sorting signals by AP complexes requiring the co-expression of two subunits from the AP-1 and AP-3 complexes. This knowledge could be helpful in the design of agents that interfere with the action of *Nef* as well as in explaining the sorting of many important cellular proteins such as the Niemann-Pick type C protein and the glucose transporter GLUT4.

Just as important as endocytosis and lysosomal targeting is the ability of cells to recycle internalized proteins to the cell surface. We have recently demonstrated that a protein named EHD1 plays a role in such a process. EHD1 cooperates with the small GTP-binding protein ARF6 to induce the formation of recycling tubules. We have also discovered that EHD1 interacts with the Rab4/Rab5 effector Rabenosyn-5. Depletion of either EHD1 or Rabenosyn-5 impairs protein recycling to the plasma membrane, demonstrating that EHD1 and Rabenosyn-5 are components of the molecular machinery for protein recycling.

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Biogenesis of lysosome-related organelles and the Hermansky-Pudlak syndrome

Martina, Moriyama, Bonifacino; in collaboration with, Ciciotte, Gwynn, Peters

The characterization of the molecular machinery involved in protein sorting is important for understanding the pathogenesis of various metabolic and developmental disorders. An example of such a disorder is Hermansky-Pudlak syndrome (HPS), a genetically heterogeneous heritable disease that affects lysosome-related organelles such as melanosomes and platelet dense bodies. We previously demonstrated that mutations in the gene encoding the beta3A subunit of AP-3 are the cause of HPS type 2. Strikingly, mutations in at least three other genes in humans and in 14 genes in mice cause a similar disorder. Most of the HPS genes identified to date by positional cloning encode proteins of unknown function and no recognizable homology to other proteins. To gain insight into the nature of this machinery, we have undertaken a biochemical characterization of the novel HPS gene products. We previously found that the protein products of the *pallid*, *muted*, and *cappuccino* genes are the components of a novel complex named BLOC-1. This past year, we demonstrated that the products of the *pale ear* and *light ear* genes are part of another complex, which we named BLOC-3. The complex is found in the cytosol and is peripherally associated with membranes. Its properties are consistent with the complex's role as a component of the molecular machinery for biogenesis of lysosome-related organelles. Ongoing studies on BLOC-1 and BLOC-3 are likely to provide additional insights into the pathogenesis of HPS.

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The pathogenesis of polycystic liver disease

Drenth, Bonifacino; in collaboration with Jansen

We recently initiated another project on polycystic liver disease, a genetic disorder related to defects in glycoprotein processing in the secretory pathway. Polycystic liver disease is a dominantly inherited condition characterized by the presence of multiple liver cysts of biliary epithelial origin. We have recently succeeded in identifying a gene that is defective in four large Dutch pedigrees of the disease. We found mutations that affect splicing of the pre-mRNA product of the gene in all four families. The gene encodes a protein that we named hepatocystin, which is predicted to be an endoplasmic reticulum protein involved in the processing of N-linked oligosaccharide chains of newly synthesized glycoproteins. We are currently investigating how defects in the protein lead to the proliferative disorder that characterizes the disease.

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REGULATION OF SECRETORY AND MEMBRANE PROTEIN BIOGENESIS

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We study the mechanisms regulating the synthesis, translocation, and maturation of secretory and membrane proteins at the mammalian endoplasmic reticulum (ER). A complex macromolecular assembly at the ER, termed the translocon, serves as a protein-conducting channel where substrates enter the secretory pathway. The translocon participates in diverse cellular activities that range from the import of secretory proteins, to topogenesis and assembly of complex multispanning membrane proteins, to the export of misfolded substrates from the ER to the cytosol for degradation. The mechanisms that allow this mutually shared translocon to accommodate such an extensive range of substrates both efficiently and accurately remain largely unknown. The principal goal of our ongoing studies is to define the molecular mechanisms and components of the translocon that recognize the information in the primary sequence of its substrates, thus mediating their proper vectorial transport, asymmetric topogenesis, and membrane integration. By delineating the steps during the biosynthesis of normal versus disease-associated variants of secretory and membrane proteins, we are formulating and testing hypotheses *in vivo* regarding the molecular basis of particular diseases of the early secretory pathway.

Molecular mechanisms of prion protein topogenesis

Kim, Fons, Hegde

The prion protein (PrP), a brain glycoprotein involved in various neurodegenerative diseases, has proven to be a particularly instructive example of complex and highly regulated translocation. In addition to its notoriety as the putative "protein-only" infectious agent in prion diseases, the biogenesis of PrP at the ER is unusual in that an initially homogeneous cohort of nascent PrP chains gives rise to three distinct topologic forms: a fully translocated form (termed ^{sec}PrP) and two transmembrane forms that span the membrane in opposite orientations (^{Ntm}PrP and ^{Ctm}PrP). *In vivo* studies have revealed that even a slight overrepresentation of the ^{Ctm}PrP topologic form results in the development of neurodegenerative disease in both mouse model systems and naturally occurring human disease. Our current efforts focus on dissecting the mechanisms that direct an initially homogeneous cohort of nascent PrP polypeptides into multiple topologic forms. We recently discovered that segregation of nascent PrP into different topologic forms is critically dependent on the precise timing of signal sequence–mediated initiation of N-terminus translocation. Consequently, this segregation step could be experimentally tuned to modify PrP topogenesis, including complete reversal of the elevated

^{Ctm}PrP caused by disease-associated mutations in the transmembrane domain. We are initiating studies in transgenic mice to determine whether ^{Ctm}PrP-mediated neurodegeneration can be averted by modulating this newly discovered step during PrP biogenesis.

Parallel biochemical studies employing the solubilization, fractionation, and reconstitution of ER membrane proteins have demonstrated that regulatory *trans*-acting factors are absolutely required for PrP to be synthesized in the proper ratio of its topologic forms. We have now purified two of these factors and identified them as the translocon-associated protein complex (TRAP) and protein disulfide isomerase (PDI). Analysis of PrP translocation intermediates suggests that TRAP and PDI act sequentially to facilitate translocation of PrP's N-terminus into the ER lumen, the decisive event in determining its topology. Ongoing studies are investigating the role of these newly discovered factors in the biogenesis of other substrates and their potential role in the pathogenesis of PrP-associated neurodegeneration.

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Modulation of prion protein cytotoxicity *in vivo*

Rane, Hegde

A principal strategy for controlling the progression of various neurodegenerative diseases is to reduce the total cellular burden of the potentially cytotoxic forms of the responsible protein. In diseases associated with the prion protein (PrP), neurodegeneration may be mediated by several variant isoforms that include a transmembrane form (termed ^{Ctm}PrP; see above) and a more recently described cytoplasmic form. To gain insight into how these variants are initially generated, we have dissected the pathway of PrP biogenesis at the ER (see above). Our results indicate that the decisive event in avoiding the generation of both of these forms is the signal sequence–mediated translocation of the N-terminus of PrP into the ER lumen. This critical step in PrP translocation is modulated by TRAP, a heterotetrameric membrane protein of previously unknown function, and ER lumenal chaperones. By using a constitutive, highly efficient signal sequence that does not depend on these factors for efficient N-terminus translocation, the generation of ^{Ctm}PrP and cytosolic PrP can be substantially reduced. The consequences of this manipulation in a cultured neuronal cell line are a marked reduction of cytotoxic and aggregation-prone variants of PrP and protection from apoptotic cell death. These results define a pathway for the normal biogenesis of PrP and demonstrate that the total cellular burden of cytotoxic forms of PrP is controlled primarily during its initial translocation into the ER. Some of our ongoing studies focus on examining the consequences in transgenic mice of altering the cellular burden of cytoplasmic forms of PrP. In particular, we wish to ask whether reducing the amounts of this potentially neurotoxic species of PrP confers protection against neurodegeneration during the course of transmissible prion disease.

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Mechanisms of calreticulin multifunctionality

Shaffer, Sharma, Hegde

The generation of multiple topologic forms of the prion protein has raised the provocative possibility that a single protein can exist in multiple spatially and functionally discrete populations. We have therefore sought to identify and characterize examples of proteins whose translocation into the ER is productively regulated to generate multiple functional forms. To this end, we have been studying the protein calreticulin, an abundant calcium-binding chaperone of the ER lumen. Remarkably, this protein has also been implicated in functions outside the ER, such as nuclear export, binding to and modulating activity of steroid hormone receptors, and binding to and influencing integrin signaling.

How this multifunctional protein might perform these functions in multiple cellular compartments remains unknown. We have recently discovered that the signal sequence of calreticulin is naturally designed in a manner that allows a small but discrete population of the protein to be retained in the cytoplasm. This cytoplasmic population can be reduced by using a signal sequence from another protein, prolactin. Thus, we have identified a potential mechanism by which a protein can be made in more than one population and a means to manipulate such heterogeneity. We are now examining the relative contributions of the different populations of calreticulin to each of the multiple functions associated with the protein. We wish to determine if and how calreticulin translocation is regulated by the cell to change its localization and, hence, function to meet changing cellular demands with stress, cell cycle, differentiation, or disease pathogenesis.

Regulation of protein biogenesis by signal sequences

Yonkovich, Mitra, Levine

Our discovery that the N-terminal signal sequence of PrP regulates its topology established a new function for this domain independent of its well-studied role in protein targeting. Using signal sequence mutants that uncouple its targeting and post-targeting functions, we demonstrated that the post-targeting function lies in gating the translocation channel to provide the nascent polypeptide access to the ER lumen. We subsequently developed and used a novel assay for signal-mediated translocon gating to demonstrate that signal sequences display remarkable variability in initiating nascent chain access to the lumenal environment. We found that such substrate-specific properties of signals are evolutionarily conserved, functionally matched to their respective mature domains, and important for the proper biogenesis of some proteins. A recent analysis of several naturally occurring disease-associated mutants in signal sequences revealed that many of them are altered in their gating function and not involved in targeting as previously assumed. Thus, we have discovered that the long-observed sequence variations of signals do not simply represent functional degeneracy but instead encode differences in translocon gating that are critical to the translocon's attached substrate. We are currently investigating whether the substrate-specific properties of signal sequences have been exploited by the cell to regulate the subcellular localization of certain proteins, such as calreticulin, that have been shown to be present in multiple compartments.

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Structural and functional analysis of the TRAP complex

Fons, Schwartz; in collaboration with Akey

The search for factors involved in PrP topogenesis led to our purification of the TRAP complex, a set of four proteins with a previously unknown function. Our recent studies establish that TRAP is required for the translocation of some, but not all, substrates and that substrate specificity is encoded in the signal sequence. Remarkably, TRAP specifically aids vectorial transport of substrates whose signal sequences, after mediating targeting to the ER, are delayed in their gating of the translocon. Thus, it appears that TRAP is a critical component of the translocation machinery that aids in decoding the substrate-specific information in signal sequences. Our current studies of TRAP function are following two approaches to understand the molecular mechanism by which it facilitates signal recognition, substrate translocation, and protein topogenesis. In collaboration with Christopher Akey's group, we are using cryo-electron microscopy to compare the structures of ribosome-translocon complexes that have been prepared with and without the TRAP complex. The studies are providing the initial structural views of not only the TRAP complex but also of its relative position within the translocon. In parallel, we are investigating the consequence *in vivo* of

RNAi-mediated suppression of TRAP expression in cultured cells. It is expected that the structural information combined with the functional analyses will provide insight into the mechanisms by which TRAP can regulate protein translocation in a substrate-specific manner.

Visualization of translocon organization in cells

Hegde; in collaboration with Lippincott-Schwartz, Snapp

A qualitatively different facet of protein biogenesis is the question of where within the ER various events occur. The translocon participates in diverse cellular activities that range from the import of secretory proteins, to topogenesis and assembly of complex multispanning membrane proteins, to the export of misfolded substrates from the ER to the cytosol for degradation. We wish to determine whether all these translocon-associated activities are homogeneously distributed throughout the ER, or whether they are organized and regulated in spatial and temporal dimensions to meet the changing needs of the cell. Currently, there is little or no insight into this question largely because the current approaches to understanding protein translocation use biochemical systems in which spatial relationships are lost. In collaboration with the laboratory of Jennifer Lippincott-Schwartz, we are using biophysical techniques such as fluorescence resonance energy transfer (FRET) to probe, *in situ,* the molecular organization of the components of the translocation machinery. In initial studies, we analyzed FRET between subunits of the Sec61p complex, a principal component of the ER protein translocon to monitor directly the assembly state of the translocon in cells. Our studies have revealed that, while the translocon can be assembled from its components in response to ligands for protein translocation in biochemical systems, it does not disassemble and reassemble between successive rounds of transport *in vivo*. Instead, an actively engaged translocon is distinguished from a quiescent translocon by conformational changes that can be directly detected by differences in FRET. By correlating the formation of particular protein complexes with biochemical activities, we endeavor to visualize directly the functional segregation and organization of the ER and to monitor potential changes in it during cellular metabolism, development, or disease pathogenesis.

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REGULATION OF MEMBRANE DYNAMICS AND PROTEIN TRANSPORT BY THE SEC7-DOMAIN GUANINE NUCLEOTIDE EXCHANGE FACTORS

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We study the Sec7-domain guanine nucleotide exchange factors (GEF) for the Arf family of small GTPases. We are interested in the roles of these proteins in membrane dynamics and protein trafficking. The Arfs and the Arf GEFs are important regulators of both organelle structure and protein transport throughout the cell. We are focusing on the large Golgi-localized Arf GEFs involved in transport through the secretory pathway both in budding yeast and mammalian cells. A central question in cell biology is how the elaborate and dynamic structures of membrane systems are maintained in the face of constant trafficking into and out of each organelle. Issues of particular interest are the way organelle structure is generated and maintained and how structure correlates with the underlying molecular events of protein sorting and membrane remodeling. An important step toward investigating these issues will be to define the roles of the Arf GEFs at the molecular level through the identification of interacting partners, elucidation of membrane localization mechanisms, and analysis of Arf GEF mutants *in vivo*.

Identification of interacting partners of the Golgi-localized Arf GEFs in yeast and mammalian cells

Smirnova, Park

The Arf GEFs of the Golgi-localized Gea/GBF and Sec7/BIG subfamilies are large multidomain proteins. A major goal of our laboratory is to understand the functions of the different domains of the Gea/GBF and Sec7/BIG Arf GEFs. Given that the GEFs are soluble proteins that must be targeted to membranes, identification of their membrane-targeting signals and partners is an important step in understanding their function. Within each subfamily, regions upstream and downstream of the Sec7 catalytic domain are conserved from yeast to humans; the functions of these homology regions are, however, not known. We are carrying out two-hybrid screens with the N-terminal and C-terminal regions of the Arf GEFs of the Gea/GBF and Sec7/BIG subfamilies, using the Sec7 domain of the mammalian homolog of Sec7p BIG2. We found a number of partners, two of which are human PI4P 5 kinase beta and a putative lipase. PI4P 5 kinase beta is a particularly interesting potential partner, as it has been identified as an effector of Arf. We carried out a reverse two-hybrid screen to determine the region of the BIG2 Sec7 domain that interacts with PI4P 5 kinase. Interestingly, we found that residues in the C-terminal region of the Sec7 domain abolish interaction with PI4P 5 kinase. We have narrowed the interaction domain of PI4P 5 kinase to a 50-amino acid region in the C-terminal portion of the catalytic domain, just upstream of the activation loop. The activation loop contains the substrate binding site and is both necessary and sufficient to determine intracellular localization of the kinase. We are also mapping the region of Arf interaction in PI4P 5 kinase. Interestingly, it appears to be in a different domain of the protein than the BIG2 Sec7 domain interacting region. It is striking that we have identified three lipid-modifying enzymes as binding partners of different Sec7 domains. It appears that all three proteins bind to the C-terminal region of the Sec7 domain. We know that this region is adjacent to a PH domain in the ARNO subfamily of Arf GEFs, which mediates membrane localization through specific lipid interaction. Using the Gea2p N-terminal region, we have identified Mss4p as an interacting partner; Mss4p is the sole PI4P 5 kinase in yeast. It is intriguing that we identified a human homolog of Mss4p in our two-hybrid screen with the BIG2 Sec7 domain. We have also identified subunits of two large complexes involved in trafficking in the early secretory pathway in two-hybrid screens with Gea2p. With the N-terminal portion of Gea2p, we identified Cog4p, a member of the eight-subunit COG complex that functions in the Golgi of both yeast and mammalian cells. In the screen with the C-terminus of Gea2p, we identified a component of TRAPP, another Golgi-localized complex conserved in all eukaryotes. These complexes have been shown to play a role in early steps of membrane fusion reactions before the actual fusion event, and we are now working toward understanding the role of the Arf GEFs in this process.

Using different regions of the Gea2 protein, we have identified six transmembrane domain proteins as potential membrane receptors for the Gea1p and Gea2p proteins in two-hybrid screens. Two of the proteins are Drs2p, a Golgi-localized amino-phospholipid translocase, and Gmh1p, a Golgi-localized five-span transmembrane protein of unknown function. As is the case for *drs2*, cells deleted for *GMH1* also show only a mild effect on Gea2p. The double mutant *drs2 gmh1* is viable, and although there is an effect on Gea2p localization that is more severe than in either single mutant, some Gea2p is still able to bind to membranes. We are testing the possibility that the other transmembrane-domain proteins identified in two-hybrid screens with Gea2p also contribute to Gea2p localization. Hence, the mechanism by which the large Arf GEFs such as Gea2p are localized to membranes appears to be complex, with multiple membrane localization determinants each contributing to the steady-state localization of the protein in cells.

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Dynamics of the GBF1 Arf GEF in mammalian cells

Niu; in collaboration with, Lippincott-Schwartz, Pfeifer

Using live cell imaging, we are studying GBF1, the mammalian homolog of Gea2p. As previously reported, a YFP-GBF1 fusion protein localizes to the Golgi in mammalian cells. Overexpression of GBF1 confers resistance to brefeldin A (BFA), a drug that profoundly affects the structure and functioning of intracellular organelles. Within 10 minutes of treatment, the Golgi apparatus is completely disassembled in a normal cell, whereas in cells overexpressing GBF1, the Golgi remains intact. YFP-GBF1 also protects cells against the effects of BFA, indicating that this fusion protein is functional. Remarkably, when cells are treated with BFA, GBF1 is recruited dramatically to Golgi membranes. We have tested different mutants in the catalytic domain of GBF1 for their response to BFA. One of these mutations, E694D, resides in the catalytic glutamic acid residue and reduces the rate of exchange 400-fold *in vitro*. There is no difference in the dynamics of GBF1-E694D in cells either before or after BFA treatment. In contrast, mutation of a residue known to affect BFA sensitivity/resistance in other Arf GEFs without affecting catalytic activity results in a version of GBF1 that is completely resistant to the effects of BFA. This result indicates that GBF1 is a direct target of BFA at the Golgi in mammalian cells. The very rapid kinetics of association-dissociation from Golgi membranes suggests that GBF1 must interact with localization machinery at each stage of the secretory pathway at which it acts rather than being recruited early in the pathway and remaining associated with membranes through successive steps of the pathway. This observation is consistent with the results described above, that is, numerous transmembrane partners have been identified for the GBF/GEA subfamily of Arf GEFs.

Role of the yeast Arf GEFs in morphology of the yeast secretory pathway

Park, Rainey, Phillips, Thomas

The Arf GEFs are important regulators of organelle structure and protein trafficking in both yeast and mammalian cells. Seemingly, yeast organelles differ substantially in structure and organization from those of mammalian cells, yet most of the proteins involved in organelle structure and trafficking, including the Arf GEFs, are highly conserved. As a first step to identifying the structural features common to yeast and mammalian organelles, our current work focuses on determining the Golgi structure in yeast by using both live imaging and electron microscopy. The use of both the yeast and mammalian systems will allow us to determine which aspects of Arf GEF function are fundamental to all eukaryotic organisms and which are unique to their specific system.

We have isolated 75 temperature-sensitive (ts) *SEC7* mutants and over 100 *GEA2* ts mutants. The mutants were generated in GFP-tagged versions of Sec7p and Gea2p and were screened by fluorescence microscopy for localization of the mutant protein at the nonpermissive temperature. We found a category of mutants in each case that showed a largely cytosolic pattern and are examining these mutants to determine whether they have lesions in a membrane localization domain. Other mutants show abnormal structures at the fluorescence level. We are currently studying these mutants at the ultrastructural level, which is providing a wealth of detailed information on the structural changes observed in the mutants not obtained by light microscopy. We sequenced all mutants to determine which amino acids were altered in the mutant proteins. A subset of the mutants have only one or two amino acid substitutions in domains highly conserved from yeast to humans. We are exploring the functions of these domains through suppressor screens aimed at identifying potential interacting partners of these regions of the Arf GEFs. Our studies will provide information on the molecular mechanisms that generate and maintain organelle structure in eukaryotic cells.

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CELL CYCLE REGULATION DURING OOGENESIS

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We use *Drosophila* oogenesis as a model to explore the developmental regulation of the cell cycle. The long-term goal of the laboratory is to understand how the cell cycle events of meiosis are coordinated with the developmental events of gametogenesis. In *Drosophila*, the oocyte develops within the context of a 16-cell germline cyst. Individual cells within the cyst are referred to as cystocytes and are connected by actin-rich ring canals. While all 16 cystocytes enter premeiotic S phase, only a single cell remains in the meiotic cycle and becomes the oocyte. The other 15 cells enter the endocycle and develop as highly polyploid nurse cells. Currently, we are working to understand how cells within the ovarian cyst enter and maintain either the meiotic cycle or the endocycle. In addition, we are examining how this cell cycle choice influences the nurse cell/oocyte fate decision.

A highly conserved nuclear protein, encoded by *missing oocyte*, is required for the maintenance of the meiotic cycle and the repair of double-stranded breaks during oogenesis

Iida, Lilly; in collaboration with Rong

To identify the pathways that direct entry into and maintenance of the meiotic cycle in the single pro-oocyte, we screened for mutants in which all 16 cells enter the endocycle and develop as nurse cells. From this screen, we identified a new gene, *missing oocyte* (*mio*), that is required for the maintenance of the meiotic cycle. In *mio* mutants, the oocyte enters the meiotic cycle, forms mature synaptonemal complexes (SC), and progresses to pachytene. However, the meiotic state is not maintained. Ultimately, *mio* oocytes abandon the meiotic cycle, enter the endocycle, and develop as nurse cells.

We characterized the molecular structure of the *mio* gene and determined that *mio* is predicted to encode a protein of 867 amino acids that is highly conserved from yeast to humans. In higher eukaryotes, all Mio family members share a similar domain structure. The amino termini contain a series of four to six well-conserved WD40 repeats. WD40 repeats often provide a surface for protein-protein interactions The WD40 repeats found in *mio* family members are most similar to those present in the chromatin-binding protein CAF1p48/RbAp48, which is a component of numerous chromatin-remodeling complexes. Specifically, CAF1p48/RbAp48 is found in complexes that modify chromatin through the acetylation and deacetylation of histones. In addition to the WD40 repeats, Mio family members contain a highly conserved 50–amino acid domain near their C termini that shares structural similarities to two well-characterized zinc-binding domains, the

RING finger and the PHD finger. RING finger domains are present in a subclass of E3 ubiquitin ligases, whereas PHD fingers have been implicated in chromatin binding. While the "Mio domain" does share structural similarities with the zinc-binding domains, it does not fit the exact consensus of either a canonical RING finger or a canonical PHD finger. Therefore, the biochemical function of this highly conserved domain remains to be determined empirically.

Mio accumulates to high levels in the oocyte nucleus during early prophase of meiosis I. Double labeling with anti-Mio antibodies and an antibody against the synaptonemal complex protein C(3)G indicates that Mio specifically localizes to the nucleus of the oocyte soon after the completion of premeiotic S phase, making Mio one of the earliest nuclear markers for the oocyte that is not a known component of the SC.

Genetic interaction studies indicate that Mio functions early in meiosis before the onset of pachytene. In *egalitarian* (*egl*) mutants, all 16-cyst cells enter the meiotic cycle and progress to pachytene, as assayed by the presence of mature SCs. However, the meiotic state cannot be maintained; eventually, all the cells exit the meiotic cycle and enter the endocycle. Intriguingly, germline cysts from *mio,egl* double mutants have a significantly stronger phenotype than either single mutant. Ovarian cysts from *mio,egl* double mutants never form mature SCs and do not progress past zygotene. The data indicate that *mio* influences the oocyte cell cycle in early prophase of meiosis before formation of the mature SC.

The *mio* ovarian phenotype is suppressed by inhibiting the formation of the double-stranded breaks (DSB) that initiate meiotic recombination during meiosis. In *mio* single mutants, the oocyte frequently enters the endocycle and becomes polyploid. However, when placed in a genetic background in which DSB formation is inhibited, the majority of *mio* egg chambers retain an oocyte and develop to late stages of oogenesis. The simplest interpretation of the data is that *mio* is required to repair the DSBs that initiate meiotic recombination and that the inability to repair DSBs significantly contributes to the *mio* phenotype. While clearly important, the inability to repair DSBs during meiosis is unlikely to be the sole cause of the *mio* phenotype. Considering that Mio localizes to the oocyte nucleus early in meiosis and functions before the construction of the mature SC, we speculate that Mio acts upstream of the enzymology of DSB repair. Thus, the inability of *mio* mutants to repair DSBs may be attributable to a general alteration in meiotic chromosome structure or, alternatively, subtle alterations in the meiotic program. Further studies of *mio* may help elucidate the poorly characterized pathways that control meiotic progression and the maintenance of oocyte identity.

The p27^{KIP1} like CDK inhibitor Dacapo regulates two variant cell cycles during the maturation of the *Drosophila* egg

Hong, Iida, Sugimura, Brown, Lilly; in collaboration with Aladjem

Animal oocytes undergo a highly conserved developmental arrest in prophase of meiosis I, often marking a period of rapid growth for the oocyte that is necessary to coordinate meiotic progression with the developmental events of oogenesis. In *Drosophila,* the oocyte develops within a 16-cell germline cyst. Throughout much of oogenesis, the oocyte remains in prophase of meiosis I. In contrast, its 15 mitotic sisters enter the endocycle and become polyploid in preparation for their role as nurse cells. How germline cysts establish and maintain these two independent cell cycles is unknown. We have shown that the p21^{CIP}/p27^{Kip1}/p57^{Kip2}-like cyclin-dependent kinase inhibitor (CKI) Dacapo maintains the prophase I meiotic arrest of the *Drosophila* oocyte. The *dacapo* gene is a vital gene that specifically inhibits the activity of CycE/Cdk2 complexes. CycE/Cdk2 activity is required for S phase in *Drosophila*. Throughout much of the growth phase of *Drosophila* oogenesis, the levels of the cki Dacapo oscillate in the 15-polyploid nurse cells but remain persistently high in the single oocyte. We have shown that both modes of Dacapo regulation are functionally important. In the oocyte, the prophase I arrest is lost, or not properly established, in germline cysts that lack Dacapo. This is the first demonstration of a cip/kip family member functioning in a normal meiotic cycle. In addition, our data indicate that Dacapo is part of the biochemical oscillator that drives the nurse cell endocycle. Specifically, we find that, in polyploid nurse cells, the oscillations of Dacapo facilitate the relicencing of DNA replication origins during endoreplication by inhibiting CycE/Cdk2 activity at the end of each endocycle S phase. Our data are consistent with recently proposed models which suggest that the periodic expression of members of the cip/kip family of Cdk inhibitors directs entry into the Gap phase during endoreplicative cycles. We propose that it is through the differential regulation of the cki Dacapo that two dramatically different cell cycles, the meiotic cycle and the endocycle, are independently maintained within the common cytoplasm of the ovarian cyst. Currently, we are performing genetic screens to identify additional genes that regulate CycE/Cdk2 during oogenesis.

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A possible mechanism for coordinating mitotic synchrony during the germline cyst divisions: the *Drosophila* fusome contains highly inter-connected endoplasmic reticulum

Iida; in collaboration with Lippincott-Schwartz

Gametogenesis in diverse organisms involves the formation of germline cysts containing interconnected germ cells. *Drosophila* ovarian cysts are produced through a series of four synchronous incomplete mitotic divisions. After each mitosis, a membranous organelle, the fusome, grows along the cleavage furrow and the remnants of the mitotic spindle to physically connect all cystocytes in the cyst. The fusome is essential for the differentiation of the oocyte, as well as for the pattern and synchrony of the mitotic cyst divisions. A long-standing goal in the field has been to elucidate the structure and origin of the fusomal membranes. We used live cell imaging, GFP, and photobleaching technology to identify definitively and characterize the endomembranes as endoplasmic reticulum (ER). Surprisingly, photobleaching revealed that fusome ER membranes are not only continuous within the fusome but also physically link the ERs of all the cystocytes within a cyst. We are currently testing the model that the interconnectivity of the cyst ER, through the fusome, drives the synchronization of the mitotic cyst divisions.

To understand more fully the origin and function of the membrane component of the fusome, we generated transgenic lines carrying a lysozyme-GFP-KDEL (lys-GFP-KDEL) chimera. In mammalian cells, the chimera is retained in the ER lumen by the COOH-terminal KDEL motif, which, in conjunction with the KDEL receptor, targets soluble proteins to the ER lumen. The targeting system is highly conserved. The *Drosophila* ortholog of the well-studied ER lumenal protein BiP contains the KDEL targeting sequence. Our observation of live germaria indicate that the lys-GFP-KDEL protein targets to a series of whip-like tubules in the cytoplasm of germline cells that are consistent with the expected location of the ER. In addition to localization in cytoplasmic structures, lys-GFP-KDEL targeted to thick branching structures that resembled fusomes. By fixing and staining the lys-GFP-KDEL–expressing ovaries with the antifusome marker Hts, we confirmed that the lys-GFP-KDEL chimera targets to fusomal membranes. The targeting of the lys-GFP-KDEL transgene to the fusome demonstrates that the fusomal membranes arise from the ER.

Electron micrographs suggest that fusomal membranes are discontinuous fragments. Reports of fragmented ER structures in other organisms are extremely rare, and live cell imaging studies have often revealed these fragmented ER structures to be fixation artifacts. To probe fusome membrane continuity, we performed FRAP (fluorescence recovery after photobleaching) of the GFP-chimeras in fusomes. Both visually and quantitatively, the GFP-chimera can be observed rapidly recovering into the photobleached region of the fusome. The fluorescence recovery occurs on both sides of the bleached region, indicating that recovery is not directional. The rapid bidirectional recovery is consistent with the fusome's structure being a single membranous compartment.

To assay the continuity of fusome and cytoplasmic ER membranes throughout the cyst, we performed FLIP (Fluorescence Loss in Photobleaching). FLIP involves repeatedly photobleaching a small region of interest. If proteins within the photobleached region are completely mobile and the entire structure is continuous with the photobleached region, then the structure's fluorescence will be depleted over time. When a small region of cytoplasmic ER from a single cystocyte was repeatedly photobleached, the ER in the entire cyst, including the fusome, rapidly lost fluorescence while adjacent cysts were unaffected. The data indicate that all cells within a single cyst share a common ER. We predict that the interconnectivity of the cyst ER, through the fusome, is important to the synchronization of the mitotic cyst divisions. To test this model, we are currently examining ER continuity in mutants that disrupt fusome structure and/or mitotic synchrony during the germline cyst divisions.

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DYNAMICS OF SECRETORY MEMBRANE TRAFFICKING, SORTING, AND COMPARTMENTALIZATION

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We investigate the global principles underlying secretory membrane trafficking, sorting, and compartmentalization within eukaryotic cells. We are using live cell imaging of green fluorescent protein (GFP) fusion proteins in combination with photobleaching and photoactivation techniques to investigate the subcellular localization, mobility, transport routes, and binding interactions of a variety of proteins with important roles in the organization and regulation of membrane traffic and compartmentalization. To test mechanistic hypotheses related to protein and organelle dynamics, we use quantitative measurements of these protein characteristics in kinetic modeling and simulation experiments. Among the topics currently under study are growth and maintenance of endoplasmic reticulum (ER) and Golgi morphology in mammalian cells and developing *Drosophila* embryos; the mechanism(s) of secretory protein transport into and out of the Golgi apparatus; membrane binding/dissociation kinetics of trafficking machinery and its regulation; the generation and maintenance of cell polarity; and organelle breakdown and reassembly during mitosis. We have also recently developed a photoactivatable GFP, whose mechanism of photoactivation is currently under investigation.

Development of a photoactivatable GFP

Patterson

By mutagenizing wild-type GFP, we have developed a variant of GFP that allows selective marking of proteins through photoactivation. The photoactivatable GFP (PA-GFP) exhibits an optical enhancement of nearly two orders of magnitude after photoactivation, making it suitable to mark specific proteins or cell populations. We are currently analyzing the mechanism of PA-GFP photoactivation, including the identification of the amino acid residues in GFP responsible for photoconversion. We are also investigating whether other photoactivatable fluorescent proteins with spectral properties that differ from those of PA-GFP can be developed to allow dual color imaging of photoactivated proteins.

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Dynamics and differentiation of the endoplasmic reticulum

Snapp

The ER mediates some of the most fundamental processes within eukaryotic cells, including the biosynthesis of protein and lipid, folding and oligomerization of proteins, lipid detoxification, calcium regulation, nuclear envelope biogenesis, and protein transport. We are using a variety of live-cell imaging techniques (i.e., FRAP, FLIP, confocal time-lapse imaging) combined with biochemical approaches to gain insight into a variety of properties of the ER membrane system. The questions we are addressing include whether proteins localized in the ER (e.g., misfolded proteins, protein translocation complexes, lumenal markers, and processing enzymes) are free to diffuse, are immobilized to a scaffold, or are actively transported within the ER and the conditions that affect these dynamics. We also are using intact and permeabilized cell systems to study the molecular basis for ER branching and fusion into an interconnected network.

One major finding from our work is the explanation for the transformation of branching ER into stacked structures called karmellae, whorls, or crystalloid ER upon overexpression of particular ER resident proteins (see cover photo and cover legend). By attaching GFP to ER-resident proteins known to induce these structures, we found that all that was required were weak homotypic interactions between cytoplasmic domains of proteins, suggesting a simple mechanism for the formation of other stacked organelles within cells.

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ER-associated degradation

Lorentz, Hailey

Many newly synthesized proteins that are misfolded or unassembled in the ER undergo retrograde translocation into the cytoplasm, where they are degraded by cytosolic proteases. To investigate the degradative pathway, we tagged photoactivatable GFP to ER-associated degradation (ERAD) substrates to visualize their fate *in vivo*. The photoactivatable (PA) GFP is ideal for such analysis in that only GFP molecules that have been photoactivated are visible within cells, allowing protein turnover to be measured in the absence of new protein synthesis. Using the PA-GFP–tagged ERAD substrates, we are studying the role of ubquitin ligases and other regulatory molecules involved in ERAD. We are also investigating the fate of ERAD substrates when proteasome activity is inhibited.

Regulation of Arf1 dynamics by Arf1GAP and Arf1GEF

Liu, Pfeifer

The cytosolic coat protein known as coatomer binds reversibly to organelle membranes to form a “coat” that mediates vesicle formation involved in cargo transport between the ER and Golgi apparatus. Binding of COPI to membranes is regulated by the small GTPase Arf1, which, in its GTP-bound state, is active and assembles coats, and which, in its GDP-bound state, is inactive and disassembles coats. We are using confocal time-lapse imaging, FRAP, and FLIP to investigate the *in vivo* dynamics of GFP-tagged versions of COPI, Arf1, ARFGAP1, and Arf1GEF. We are exploring whether membrane binding and release of COPI initiate and stabilize cargo sorting into membrane domains and are studying the functional interaction of ARFGAP1 (which controls how rapidly Arf1 is released from membranes) with Arf1 and COPI on Golgi membranes. We also are using kinetic analysis tools of computational cell biology to test different models of cargo transport mediated by COPI/Arf1 machinery. We are testing one model that incorporates reversible partitioning of cargo into membrane domains through repeated cycling of Arf1 and COPI onto Golgi membranes to retrieve retrograde cargo and its associated lipids, thus promoting the partitioning of anterograde cargo into sphingolipid-rich domains.

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Protein trafficking through the Golgi apparatus

Hirschberg, Patterson

We have developed a protocol involving the selective highlighting of GFP-tagged cargoes in the Golgi and the monitoring of the distribution and export kinetics of these cargo molecules as they pass through the Golgi apparatus. Using this approach combined with conventional biochemical and ultrastructural methods, we are seeking to obtain new information regarding the mechanism of transport of proteins through the Golgi apparatus. We aim to determine whether the numerous cisternae comprising the Golgi act as cargo carriers in a process of cisternal maturation or whether the cisternae are stable elements with cargo transferred between them by small vesicles or by direct connections.

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ER and Golgi dynamics in the developing *Drosophila* embryo

Frescas, DeLotto

The syncytial blastoderm *Drosophilia* embryo provides a unique model system for studying changes in the organization and dynamics of the ER and Golgi apparatus in a voluminous, multinucleated system during development. To follow ER and Golgi dynamics in this system, we have expressed GFP-tagged organelle markers, lysosome-KDEL (ER), and galactosyltransferase (Golgi) in living *Drosophila* embryos and performed time-lapse confocal microscopy. We investigated the following: (1) the distribution of ER and Golgi within the embryo before cellularization; (2) the linkage of their organization to individual nuclei; (3) the dependence of the linkage on microtubules and actin; and (4) the mechanism of secretion within this voluminous system and whether it occurs in a polarized fashion.

Dynamics of lipid rafts at the cell surface

Kenworthy

Recent studies have provided insight into the organization of raft microdomains in cell membranes and suggest that the microdomains are small and discrete. Nevertheless, many of the fundamental properties of lipid rafts remain ambiguous. It is still unclear whether rafts are stable structures, how long they live, to what extent proteins move into and out of them, and whether rafts themselves are free to diffuse across cell membranes. To address these questions, we are using confocal FRAP to study the dynamics of GFP-tagged, raft-associated proteins. By comparing the diffusional mobility of several types of raft and nonraft proteins, we aim to distinguish between different models for the organization and dynamics of lipid rafts.

Delivery of apical and basolateral proteins

Polishchuk, Arias*

The generation and maintenance of cell polarity requires protein segregation into apical and basolateral plasma membrane domains. The process is thought to occur either by protein sorting into separate exocytotic routes at the TGN or by transcytosis from the basolateral to apical surface. We are using live cell imaging approaches to study the pathways and machinery involved in the generation of apical and basolateral surface domains in polarized MDCK (kidney) and Wif B (liver) cells. We are following the transport routes of different cargo proteins, including VSVG-

CFP, GPI-YFP, and GFP-tagged ABC transporters, after export from the Golgi apparatus upon shifting the temperature from 20°C to 32°C. The proteins are being imaged in polarized cells, using photoactivatable GFP, which allows the selective highlighting and tracking of the proteins in the absence of any other signal within the cell. We are using the expression of mutant proteins and pharmacological manipulations to identify the molecular machinery (e.g., rab proteins, t/v SNAREs, rafts) regulating the segregation of apical and basolateral proteins in polarized cells.

Arf1 regulation of mitotic Golgi disassembly

Altan-Bonnet

In mitosis, chromosome, cytoskeleton, and organelle dynamics must be coordinated for successful cell division. We are currently investigating how such coordination occurs and have discovered a major role of Arf1, a small GTPase associated with the Golgi complex. Our data suggest that Arf1 helps orchestrate mitotic Golgi breakdown, chromosome segregation, and cytokinesis. We found that, early in mitosis, Arf1 becomes inactive and dissociates from Golgi membranes, followed by the dispersal of numerous Arf1-dependent peripheral Golgi proteins, including cullins, myosin IIa, tankyrase, spectrin, ankyrin, and Sak1 polo-like kinase, with roles in coordinating mitotic processes. If Arf1 is kept in an active state by treatment with the small molecule H89 or expression of its GTP-locked form, intact Golgi membranes with bound peripheral proteins persist throughout mitosis. The cells enter mitosis but exhibit gross defects in chromosome segregation and cytokinesis. Our findings suggest that mitotic Golgi disassembly is dependent on Arf1 inactivation and is used by the cell to disperse numerous peripheral Golgi proteins for coordinating the behavior of Golgi membranes, chromosomes, and cytoskeleton during mitosis. We propose that Arf1 serves as a cell cycle regulator to coordinate Golgi dynamics with other cellular functions.

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REGULATION OF INTRACELLULAR IRON METABOLISM

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Previously, our laboratory identified and characterized the *cis* and *trans* elements that regulate iron-dependent alterations in the expression of ferritin and the transferrin receptor. Iron-responsive elements (IREs) are RNA stem-loops found in the 5′ end of ferritin mRNA and the 3′ end of transferrin receptor mRNA. We have cloned, expressed, and characterized two essential iron-sensing proteins, Iron Regulatory Protein 1 (IRP1) and Iron Regulatory Protein 2 (IRP2). IRPs bind IREs when iron levels are depleted, resulting in (1) the inhibition of translation of ferritin mRNA and other transcripts with IREs near the 5′ end and (2) the prolongation of the half-life of the transferrin receptor mRNA.

Iron-sulfur cluster assembly

Tong, Brazzolotto

IRP1 is an iron-sulfur protein related to mitochondrial aconitase, a citric acid cycle enzyme that functions as a cytosolic aconitase in cells that are iron-replete. Regulation of the RNA binding activity of IRP1 involves a transition from a form of IRP1 in which a [4Fe-4S] cluster is bound to a form that loses both iron and aconitase activity. The [4Fe-4S]-containing protein does not bind to IREs, and the status of the cluster appears to determine whether IRP1 will bind to RNA. Recently, we have identified mammalian enzymes of the iron-sulfur cluster assembly that are homologous to the NifS and Nif U genes implicated in bacterial iron-sulfur cluster assembly and have shown that these gene products facilitate assembly of the iron-sulfur cluster of IRP1. We have discovered that single genes in the human genome encode mitochondrial and cytosolic forms of the cysteine desulfurase IscS and the proposed scaffold proteins IscU and NFU. NFU, a protein that assembles a [4Fe-4S] cluster, is an abundant protein in mitochondria and the cytosol. NFU may function as a scaffold for iron-sulfur cluster assembly by donating its newly assembled clusters to recipient proteins.

Tong WH, Jameson GN, Huynh BH, Rouault TA. Subcellular compartmentalization of human Nfu, an iron-sulfur cluster scaffold protein, and its ability to assemble a [4Fe-4S] cluster. *Proc Natl Acad Sci USA* 2003;100:9762-9767.

Iron-dependent degradation of IRP2 and other proteins

Ghosh

IRP2 also binds to IREs in iron-depleted cells but, unlike IRP1, IRP2 is degraded in cells that are iron-replete. Experimental evidence indicates that IRP2 undergoes iron-catalyzed oxidation. The oxidized protein is then selectively ubiquitinated and degraded by the proteasome. Indirect evidence suggests that numerous other proteins are degraded by a pathway in which oxidative modification is followed by ubiquitination and proteasomal degradation of the ubiquitinated substrate. Heme is implicated in IRP2 degradation, but it is not yet clear whether free heme directly oxidizes IRP2 or if heme is a cofactor for a *trans*-acting factor involved in iron-dependent degradation.

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Physiology and regulation of iron metabolism

Smith, Cooperman, Grabill, Jui-chen, Meyron-Holtz, Land, Missirlis

To approach questions about the physiology of iron metabolism, we have generated loss-of-function mutations of IRP1 and IRP2 in mice through homologous recombination in embryonic cell lines. In the absence of provocative stimuli, we observed no abnormalities in iron metabolism associated with loss of IRP1 function. IRP2$^{-/-}$ mice develop a progressive movement disorder characterized by gait abnormalities and tremor. Animals accumulate iron in axons and develop axonal degeneration. Ferritin overexpression occurs in affected neurons, and ferritin accumulation occurs in axons. These findings are greatly accentuated in animals that lack one copy of IRP1 in addition to both copies of IRP2. IRP2 is the predominant regulator of posttranscriptional iron metabolism in animals, but IRP1 also contributes to baseline regulation. Ferritin iron accumulations in the brain can be detected on magnetic resonance imaging. Vacuolar changes that develop as a result of neuronal cell body loss in regions such as the substantia nigra can be detected on MRI and pathology. Animals that lack both IRP1 and IRP2 do not survive past the blastocyst stage.

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Structural characterization of IRPs and IREs

Yikilmaz

We have purified milligram quantities of IRP1 and IRP2 and are working on crystallization of each IRP. In addition, we are trying to cocrystallize each IRP in a complex with IRE. We have characterized and overexpressed an IRP-like protein from *Plasmodium falciparum*. To ensure that high-quality IRP is used in cocrystallization experiments, we developed a novel RNA affinity column that purifies IRP and removes protein that is unable to bind to IREs.

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MICROBIAL RESPONSES TO OXIDATIVE STRESS AND NONCODING RNAS

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The Section on Environmental Gene Regulation has two main interests: (1) the characterization of the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress and transcriptional regulators whose activities are modified by oxidation and reduction; and (2) the identification and characterization of all *E. coli* noncoding RNAs.

Microbial responses to oxidative stress
Storz, Andrade, Carmel-Harel, Mukopadhyay, Outten, Wood
One focus of the group is to study how organisms sense environmental signals and transduce the signals into changes in gene expression and cell physiology. Specifically, we are examining the *E. coli* and *S. cerevisiae* responses to oxidative stress. Reactive oxygen species can lead to the damage of almost all cell components (DNA, lipid membranes, and proteins) and have been implicated as causative agents in several degenerative diseases. Most organisms have an adaptive response to defend against oxidants. For example, treatment of both bacterial and yeast cells with low doses of hydrogen peroxide results in the induction of a distinct group of proteins, the decreased expression of other proteins, and resistance to killing by subsequent higher doses of hydrogen peroxide.

In bacterial cells, the pivotal regulator of the inducible defenses against hydrogen peroxide is the OxyR transcription factor. We discovered that OxyR is both the sensor and transducer of the oxidative stress signal; the oxidized but not the reduced form of the purified regulator can activate transcription *in vitro*. OxyR is activated by the formation of an intramolecular disulfide bond between C199 and C208 and is deactivated by enzymatic reduction by glutaredoxin 1 together with glutathione. Structural studies showed that formation of the C199-C208 disulfide bond leads to a large conformational change. Computational and microarray experiments allowed us to identify many of the genes regulated by OxyR. We now are examining the chemical basis of OxyR sensitivity to hydrogen peroxide and the roles of all of OxyR target genes.

Compared with the bacterial response to hydrogen peroxide, less is known about the cellular mechanisms used by higher cells to sense and protect against oxidative damage. To initiate studies of the oxidative stress response in eukaryotes, we constructed isogenic *S. cerevisiae* strains carrying mutations in known signal transduction pathways and compared the oxidant sensitivities and whole genome expression patterns of these mutants. The studies confirmed that the Yap1 transcription factor is critical for resistance to hydrogen peroxide. We have purified the Yap1 protein and have begun biochemical experiments to characterize this redox-sensitive transcription factor. Mass spectrometry analysis revealed that the oxidized form of Yap1p contains two disulfide bonds between C303-C598 and C310-C629. Upon limited proteolysis of oxidized but not reduced Yap1p, we detected a stable domain of about 15 kDa. We purified the Yap1p protease-resistant domain, and mass spectrometry analysis showed that it comprises two separate cysteine-containing peptides of Yap1p. The peptides are separated by 250 amino acids and are joined by the C303-C598 and C310-C629 disulfide bonds. Work to determine the structure of this modular redox domain now is under way.

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Noncoding RNAs

Storz, Kawano, Opdyke, Reynolds, Zhang; in collaboration with Gottesman, Rosenow, Tjaden, Wassarman

A second focus of the group is to identify untranslated, regulatory RNAs and to elucidate their functions. These noncoding RNAs have been shown to have roles in transcriptional regulation, chromosome replication, RNA processing and modification, mRNA stability and translation, and even protein degradation and translocation. We have been characterizing two previously identified *E. coli*–regulatory RNAs, OxyS and 6S RNA. The OxyS RNA, whose expression is induced by OxyR in response to oxidative stress, acts as (1) a global regulator that activates and represses the expression of multiple genes and (2) an antimutator that protects cells against DNA damage. Studies of the *fhlA* and *rpoS* targets showed that the OxyS RNA represses translation of these genes. We recently found that OxyS RNA action is dependent on the Sm-like Hfq protein and that Hfq functions as a chaperone to facilitate OxyS RNA base pairing with its target mRNAs. We also discovered that the abundant 6S RNA binds to and modifies RNA polymerase.

Most genome annotation has missed noncoding RNA genes, which are usually poor targets in genetic screens and thus have proven difficult to detect by direct sequence inspection. Accordingly, we have been carrying out systematic screens for additional noncoding RNA genes in *E. coli*. The screens are all applicable to other organisms. One approach based on computer searches of intergenic regions for extended regions of conservation among closely related species has led to the identification of 17 conserved noncoding RNAs. Another screen for noncoding RNAs that coimmunoprecipitate with the Hfq RNA binding protein allowed us to detect six less well conserved RNAs. A third approach involving size fractionation of total RNA followed by linker ligation and cDNA synthesis has led to the cloning of nine antisense RNAs. Studies to characterize the functions of the newly identified RNAs are ongoing.

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NEUROENDOCRINOLOGY OF STRESS

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The goal of the laboratory is to understand the neuroendocrine mechanisms underlying the stress response, with emphasis on the regulation of the hypothalamic pituitary adrenal (HPA) axis. The ability of the organism to adapt to acute and chronic stress situations is determined by genetic constitution and previous experiences. Our studies have shown that exposure to a repeated somatosensory stress causes hyperresponsiveness of the HPA axis to a novel stress. Given that hyperactivity of the HPA axis has been implicated in the pathogenesis of a number of psychiatric and metabolic disorders, self-limitation of the stress response is critical to avoiding deleterious effects of glucocorticoid excess. We study the mechanisms by which the expression of the hypothalamic hormones corticotropin releasing hormone (CRH) and vasopressin (VP) and their pituitary receptors are regulated under different stress situations and the consequences of such regulation on ACTH secretion and adrenal steroidogenesis

Regulation of hypothalamic CRH and VP expression

Kalintchenko, Nikodemova, Liu, Aguilera

Our studies have been pivotal in leading to an understanding of the role of the interaction between CRH and VP in the regulation of pituitary ACTH and of the expression of these peptides in the PVN during stress and other alterations of the HPA axis. Previous studies showed that CRH and VP co-expressed in the same parvocellular neuron of the paraventricular nucleus (PVN) are differentially regulated during stress or exposure to glucocorticoids. VP becomes the predominant peptide expressed in parvocellular neurons of the PVN during chronic stress. However, our studies suggest that, despite the prevalence of VP, ACTH secretion depends primarily on rapid but limited increases of CRH secretion and transcription. During the past year, we have focused on the elucidation of molecular mechanisms of regulation of CRH expression by using recently characterized hypothalamic cell lines with a parvocellular neuron phenotype.

We used reporter genes to investigate the influence of regions downstream of the coding region of the CRH gene on cAMP-dependent CRH gene expression. We showed that the addition of about 700 bp of the 3′untranslated region (UTR) downstream of luciferase markedly potentiated forskolin-stimulated CRH promoter activity in hypothalamic cells. Interestingly, the 3′UTR of CRH did not enhance but rather inhibited forskolin-stimulated transcription, as shown by nuclear run-on assays and the lack of increase in luciferase mRNA after forskolin stimulation in the presence of the 3′UTR. Using tetracycline-inducible constructs, we showed that stimulation of translation is a major mechanism by which forskolin stimulates CRH expression and that the 3′UTR potentiates this effect. Stimulation of cAMP by forskolin also increased Luc mRNA stability regardless of the presence of the CRH 3′UTR in the construct. The data show that cAMP stimulates CRH expression at multiple loci, including transcription, mRNA stability, and mRNA translation. In addition, distal downstream elements in the CRH gene appear to influence regulation of gene expression by inhibiting transcription and enhancing cAMP-stimulated translation.

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Self-limitation of stress responses

Nikodemova, Shepard, Aguilera

We are conducting *in vivo* and *in vitro* studies to determine mechanisms responsible for the termination of the stress response. A recognized negative feedback during the HPA axis response is the effect of increased circulating glucocorticoids in the brain and pituitary. However, stress causes refractoriness to the inhibitory effect of glucocorticoids and leads to ineffectiveness of the feedback mechanism. We are elucidating the molecular mechanisms modulating the effectiveness of glucocorticoid feedback as well as the role of both neurotransmitters such as GABA and autoregulatory mechanisms in hypothalamic neurons in limiting HPA axis responses to stress.

While CRH is essential for stress response, our studies have shown that the increases in CRH transcription during stress are transient, even if the stimulus is sustained. Using adrenalectomized rats with replacement corticosterone levels kept constant, we have shown that termination of CRH transcription occurs independently of the increases in plasma glucocorticoids in response to stress. We also showed that termination of CRH transcription is associated with increased expression of inducible isoforms of the cAMP-responsive element modulator (CREM), such as inducible cAMP early repressor (ICER), which are known to inhibit cAMP-induced transcription. During the past year, we used the CRH-expressing hypothalamic cell lines H32 and 4B and showed that transfection of ICER markedly inhibits cAMP-induced stimulation of CRH promoter activity after co-transfection with a luciferase reporter gene driven by a CRH promoter fragment. Western blot analysis of nuclear extracts of cells incubated with forskolin revealed time-dependent increases in ICER. This paralleled formation of ICER-CRH CRE complexes in the electromobility gel shift assay (EMSA), suggesting that endogenous levels of ICER can interact with the CRH CRE. EMSA using hypothalamic nuclear extracts of control and stressed rats and CRH CRE–radiolabeled oligonucleotides showed several shifted bands, of which the two lower bands were supershifted with CREM antibody.

Given that upstream and downstream elements in the gene influence promoter activity, we developed a system to study transcriptional regulation of the endogenous gene in the hypothalamic cell line 4B. Although 4B expresses endogenous CRH, transfection with the full CRH gene enhanced expression. Quantitative RT-PCR using primers directed to the CRH intron revealed transient increases in CRH heteronuclear (hn) RNA or primary transcript, followed by a declining phase concomitant with increases in ICER protein. Interestingly, in contrast to the marked inhibition of CRH promoter activity by co-transfection of CRH promoter–driven reporter genes with the repressor forms of CREM (ICERI, ICERII, CREM beta), transfection of ICER I caused only minor inhibition of forskolin-stimulated CRH hnRNA, suggesting that the inhibitory effect of ICER on

CRH promoter–driven luciferase activity is partly mediated by post-transcriptional mechanisms. The data indicate that intracellular feedback mechanisms mediated by the induction of repressor isoforms of CREM may contribute to the termination of CRH transcription in parvocellular neurons of the PVN. Current efforts are directed to establishing stably transfected cell lines with the CRH gene and studying the interactions between cAMP, other signaling pathways, and glucocorticoids on the regulation of CRH transcription.

Neuroendocrine immune interactions

Grinevich, Knepper, Verbalis, Aguilera

Our work has led to important findings on the effects of immune challenge on neuroendocrine responses in normal rats and in an experimental model of autoimmune arthritis. Acute or repeated administration of lipopolysaccharide (LPS) leads to marked activation of the HPA axis with activation of parvocellular neurons of the PVN and increases in plasma ACTH and corticosterone. Despite exacerbation of these effects in animals with chronic arthritis, we have observed that the animals experience much higher peripheral and central immunological response to challenge with LPS. We plan studies that will investigate the mechanism by which chronic inflammatory disorders can influence the responses to a novel immune challenge.

In contrast to the HPA axis, LPS has only minor effects on basal activity of the hypothalamic neurohypophyseal axis. The effects include minor decreases in VP mRNA in magnocellular neurons of the PVN and plasma VP levels within six hours of LPS injection, suggesting that immune stimulation may affect the central regulation of fluid balance. However, experiments in rats subjected to osmotic stimulation showed that endotoxemia potentiates rather than inhibits magnocellular neuron responses to osmotic challenge. Despite unchanged or elevated plasma vasopressin (VP) levels, the presence of fluid metabolism alterations suggests that endotoxemia causes refractoriness of the kidney to the effect of the peptide. To test this hypothesis, we examined the effect of LPS injection on plasma VP, urine osmolality, and the expression of V2 receptors and aquaporin-2 in the kidney. LPS injection caused prolonged decreases in urine osmolality without significant changes in plasma levels of sodium or VP, which were associated with marked decreases in V2 VP receptor mRNA measured by *in situ* hybridization, and VP binding to kidney medulla membranes. Aquaporin-2 in kidney inner medulla, measured by immunohistochemistry and Western blot, also decreased. These changes paralleled marked increases in cytokine expression in the kidney medulla. In addition, *in vitro* incubation of kidney medulla slices with IL-1 beta reduced VP binding, suggesting that cytokines are at least in part responsible for the marked downregulation of V2 VP receptors and aquaporin-2 of the kidney inner medulla observed during LPS induced endotoxemia.

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Regulation of pituitary CRH and V1b VP receptors

Volpi, Young, Aguilera

Regulation of the number of CRH and VP receptors in the pituitary plays an important role in the control of HPA axis activity. Our laboratory has completed studies showing that CRH receptor content in the pituitary does not depend on the levels of CRHR1 mRNA, indicating that regulation of the number of functional receptors occurs at post-transcriptional sites. During the past year, we focused on the regulation of the V1b VP receptor, which is the major subtype in the pituitary corticotroph. We demonstrated that increased pituitary corticotroph responsiveness during chronic stress is associated with VP receptor upregulation. Studies on the transcriptional regulation of the V1b VP receptor have identified a region in the proximal promoter containing a large GAGA repeat, which is essential for transcriptional activation of the V1b receptor promoter and binds to a protein complex found in pituitary nuclear extracts. Stress causes transient increases in GAGA binding activity of pituitary nuclear extracts, an effect possibly mediated by VP. We used the hypothalamic cell line H32, which expresses endogenous VP receptors, to study the signaling pathways responsible for regulation of GAGA binding activity. In these cells, VP stimulated GAGA binding activity, an effect that was mimicked by epidermal growth factor (EGF) and blocked by inhibitors of the EGF receptor and mitogen-activated protein kinase (MAPK) pathway, suggesting that VP activates V1b receptor transcription through transactivation of the EGF receptor. A novel feature of the regulation is that, in contrast to other systems, protein kinase C inhibition potentiates VP-induced MAPK activation as well as the effects of VP on GAGA binding activity, perhaps indicating that PKC is inhibitory and that stimulation of PKC is part of the mechanism for termination of MAPK signaling in neurons. Using receptor subtype–specific analogs or different cell lines transfected with either V1a or V1b receptors, we showed that the pathways involved in transactivation of the MAPK pathway by VP are cell-specific and independent of the V1 receptor subtype. We are now characterizing the GAGA binding protein complex and the role of other transcription factors on the regulation of the V1b receptor gene.

Previous studies conducted in our laboratory point to a lack of correlation between V1b receptor mRNA and V1b receptor binding, suggesting that regulation at post-transcriptional sites plays an important role in determining the number of VP receptors in the pituitary. During the past year, we showed that the 5′UTR of the V1b receptor mRNA plays an important role in controlling translation of the mRNA into receptor protein. The presence of upstream open reading frames in the 5′UTR may play a role maintaining low translational activity in basal conditions. On the other hand, during the past year, we identified an internal ribosome entry site (IRES) in the 5′UTR. IRES activity can be stimulated by activation of protein kinase C and PI3 kinase–dependent pathways. The data suggest mechanisms by which the 5′UTR of the V1b mRNA can determine negative and positive regulation for translation of V1b receptor mRNA according to physiological requirements.

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REGULATION OF SKELETAL GROWTH

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We investigate the cellular and molecular mechanisms governing bone growth and development. One goal of our work is to improve medical treatment of growth disorders and childhood metabolic bone diseases. In addition, given that the cellular processes underlying bone growth, such as cell proliferation, terminal differentiation, angiogenesis, and cell migration, are also essential for development in other tissues, we seek to uncover general principles of developmental biology.

Longitudinal bone growth: cellular and molecular mechanisms

Baron, Barnes, Nilsson, Gafni, Nwosu, Mitchum

Longitudinal bone growth occurs at the growth plate, a thin layer of cartilage that lies near the ends of long bones and vertebrae. The growth plate consists of three principal layers: the resting zone, the proliferative zone, and the hypertrophic zone. Studies in our laboratory indicate that the resting zone contains stem-like cells that are capable of generating new clones of proliferative chondrocytes. We have reported evidence that the resting zone directs the spatial orientation of the proliferative clones, causing them to form columns parallel to the long axis of the bone. These proliferative cells undergo clonal expansion followed by cellular hypertrophy. The hypertrophic cartilage is then remodeled into bone tissue. The net effect is the progressive creation of new bone tissue at the bottom of the growth plate, resulting in bone elongation. This process of endochondral ossification at the growth plate results in the formation of new trabecular bone. However, longitudinal bone growth requires the formation not only of trabecular bone (spongy bone found in the interior of long bones) but also of cortical bone (dense bone found at the periphery of long bones). The mechanisms responsible for longitudinal growth of the bone cortex have not previously been identified. We considered two alternative hypotheses: trabecular cortical bone could be formed by either (1) coalescence of trabecular bone formed at the periphery of the growth plate or (2) intramembranous bone formation (i.e., without a cartilage intermediary) at the periosteal surface (outer surface of the bone). We therefore undertook a study to distinguish between the two hypotheses and to explore the underlying cellular and molecular mechanisms.

The results of our study indicate that longitudinal growth of the cortex occurs by coalescence of trabecular bone formed at the periphery of the growth plate. Three lines of evidence support our conclusion. First, to prevent the passage of cells, we surgically inserted a semipermeable membrane in the metaphysis of growing rabbits, between the periosteum and underlying trabecular bone of the proximal tibias. We observed that, despite its separation from the overlying periosteum, the trabecular bone transformed into cortical bone, thus demonstrating that metaphyseal cortical bone was not formed by subperiosteal bone deposition. Second, we observed remnants of cartilage within the forming metaphyseal cortex, indicating that this region is generated by endochondral bone formation. Third, we repetitively administered oxytetracycline to growing rabbits to label newly synthesized bone. In the diaphyseal (midshaft) cortex, we observed fluorescent lines parallel to the periosteum consistent with growth by subperiosteal apposition. In contrast, in the metaphyseal cortex (i.e., closer to the growth plate), we observed fluorescent closed curves outlining enlarging trabeculae. Thus, in the metaphysis, trabeculae generated by endochondral bone formation at the periphery of the growth plate enlarge and coalesce to create new cortical bone. In this region of coalescing trabeculae close to the periosteum, the osteoblast surface (the fraction of bone surface covered by osteoblasts) increased as compared with trabeculae farther from the periosteum. The osteoclast surface did not differ. *In vitro*, proliferation increased when osteoblasts were cultured in the presence of periosteum or periosteum-conditioned medium. Surgical insertion of permeable or impermeable membranes between periosteum and spongiosa did not prevent cortex formation.

Based on these studies, we conclude that metaphyseal cortical bone is formed by coalescence of endochondral trabecular bone. The coalescence is associated with increased osteoblast surface in the peripheral trabecular bone. The increased osteoblast surface could be attributable to the inductive effects of periosteum; periosteum stimulates osteoblast proliferation *in vitro* but is not required for metaphyseal cortical bone formation *in vivo*.

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Regulation of skeletal growth by estrogen

Baron, Barnes, Nilsson, Gafni

The rate of longitudinal bone growth declines drastically with age. As a result, in humans, the linear growth rate (change in body length per year) decreases from over 100 cm per year *in utero* to 50 cm per year at birth, 5 cm per year by mid-childhood, and after the pubertal growth spurt 0 cm per year in late adolescence. A similar progressive decline in bone growth occurs in other mammals. Studies in our laboratory suggest that the decrease in longitudinal bone growth occurs because the growth plate stem-like cells have a finite proliferative capacity that is gradually exhausted. The cellular and molecular mechanisms that limit proliferation of growth plate chondrocytes are currently under investigation. Eventually, growth ceases and the growth plate is replaced by bone, a process known as epiphyseal fusion. Our findings suggest that epiphyseal fusion is triggered when the proliferative capacity of the growth plate chondrocytes is exhausted. We have found evidence that estrogen accelerates the proliferative exhaustion of the growth plate chondrocytes. As a result, estrogen leads to early termination of linear growth and early epiphyseal fusion. Consistent with this hypothesis, we have found that estrogen alpha and beta receptors are both expressed in growth plate chondrocytes throughout postnatal development in both rats and rabbits.

We have recently completed a clinical study to determine whether estrogen accelerates proliferative exhaustion in human growth plate chondrocytes. We analyzed growth data from girls with precocious puberty treated with a gonadotropin-releasing hormone analog (GnRHa). In these girls, the precocious puberty caused the growth plates to be exposed to high levels of estrogen. Our animal studies suggest that such estrogen exposure would cause accelerated proliferative exhaustion of growth plate chondrocytes. Treatment with GnRHa causes normalization of estrogen. However, if the proliferative capacity of the growth plate chondrocytes has been exhausted by the previous estrogen exposure, we would expect the growth rate to be low during GnRHa treatment. Our model also predicts that the height velocity during treatment would be inversely related to the severity of earlier estrogen exposure. To test these predictions, we analyzed data from 100 girls (age 5.8 ± 2.1 years, mean ± SD) with central precocious puberty treated with GnRHa. During GnRHa therapy, height velocity was low for age (-1.6 ± 1.7 SD score, mean ± SD). The absolute height velocity correlated most strongly with the bone age, a surrogate marker for growth plate senescence. The severity of the growth abnormality (height velocity SD score for age) correlated inversely with markers of the severity of earlier estrogen exposure, including duration of precocious puberty, Tanner breast stage, and bone age advancement. Stepwise regression confirmed that bone age was the best independent predictor of growth during GnRHa therapy. These findings are consistent with our hypothesis that estrogen accelerates proliferative exhaustion in human growth plate chondrocytes.

Estrogen treatment has been used clinically to induce growth plate fusion, thereby reducing the final height in girls expected to achieve extreme tall stature. The treatment is effective in terms of limiting final height, but it raises concerns about possibly increasing the risk for malignancies later in life. Raloxifene, a selective estrogen receptor modulator, has been shown to act as an estrogen agonist on bone density but as an estrogen antagonist on breast and uterine tissue. The effect of raloxifene treatment on growth plate fusion and final height was unknown. We therefore undertook a study to determine whether raloxifene would act as an estrogen agonist or antagonist on growth plate cartilage. We treated ovariectomized immature rabbits for four weeks with vehicle (controls), estradiol cypionate (E2), or raloxifene. Tibial growth velocity declined in both E2- and raloxifene-treated animals as compared with controls. E2 and raloxifene treatment also decreased chondrocyte proliferation and the height of the proximal tibial growth plate. In addition, E2 and raloxifene hastened fusion of the distal tibial growth plate and decreased the number of proliferative and hypertrophic chondrocytes per column in the proximal tibial growth plate. As expected, the uterus was enlarged by treatment with estrogen but not raloxifene. We concluded that raloxifene acts as an estrogen agonist on the growth plate, accelerating growth plate senescence and thus hastening epiphyseal fusion.

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Clinical studies

Gafni, Leschek, Baron

We have completed a randomized double-blind placebo-controlled trial of growth hormone (GH) therapy in children with marked idiopathic short stature. Currently, thousands of children with severe idiopathic short stature receive growth hormone therapy despite the absence of definitive data regarding long-term efficacy and safety. Nonrandomized long-term studies have yielded conflicting results as to whether growth hormone therapy increases the adult height of children without growth hormone deficiency. As early as 1983, researchers saw GH therapy for children without GH deficiency as a public health issue; the "Conference on Uses and Possible Abuses of Biosynthetic Human Growth Hormone," convened by the NICHD, concluded that "there is an urgent need for therapeutic trials to determine the effect of growth hormone in short children who do not have a growth hormone deficiency." Similarly, in 1987, the FDA Endocrinologic and Metabolic Drugs Advisory Committee called for well-controlled studies on the long-term safety and efficacy of growth hormone for children who are not growth hormone–deficient. In response, the NICHD initiated a randomized, double-blind, placebo-controlled trial to determine the effect of GH on adult height in such children. During the course of the study, 68 children, 9 to 16 years old, with marked, idiopathic short stature (height or predicted height $\leq$ -2.5 SDS, i.e., $\leq$ 0.6 percentile) received either GH or placebo until they were near adult height. At study termination, adult height measurements were available for 33 patients after a mean treatment duration of 4.4 years. Adult height was greater in the GH-treated group *(-1.81* ± 0.11 SDS, least squares mean ± SEM) than in the placebo-treated group (-2.32 ± 0.17 SDS) by 0.51 SDS (3.7 cm, $p < 0.02$, 95% CI 0.10, 0.92 SDS). Intent-to-treat analyses indicated a similar GH effect and no important dropout bias. The results indicate that GH treatment increases adult height in children with marked idiopathic short stature. However, at least for the regimen used in the current study, the effect was modest. These observations do not imply that GH should be used routinely to treat children with short stature. To the contrary, mild short stature appears to have only mild psychological consequences and is usually not treated medically. Even for children with more extreme short stature, similar to the GH-deficient phenotype, any benefit derived from an increase in height must be weighed against the risk of adverse events, the cost, and the discomfort of GH injections.

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GENES OR HORMONES? GENDER-SPECIFIC DIFFERENCES IN DISEASE SUSCEPTIBILITY

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Judith Ross, MD, *Guest Researcher*[d]

In addition to the obvious phenotypic differences between the sexes, there are gender-specific differences in longevity and cognitive, immunological, and metabolic functions. Our goal is to elucidate the genetic and physiological mechanisms underlying these important but poorly understood differences. The genetic contribution to sexual dimorphism was initially believed to begin and end with the action of genes specifying gonads during early embryonic development. Such a belief was premised on the assumption that only one X-chromosome was active in females and that the Y-chromosome's unique contribution consisted of testis-determining genes such that all phenotypic differences occurring subsequent to gonadal development were attributed to sex steroid effects. We now realize that many X-chromosome genes escape inactivation and may contribute to gender differences by dosage effects. We aim to identify and define the function of X-chromosome genes involved in the differential development and function of brain, reproductive, metabolic, and immune systems in women and men. The study of monsomy X, or Turner's syndrome, provides a unique opportunity to elucidate X-chromosome gene dosage effects. This research will enhance our understanding of disease processes such as the increased susceptibility to autoimmune disease in women and increased risk for coronary disease in men.

X-chromosome genes and coronary heart disease

Bakalov, Hanton, Cooley, Axelrod, Bondy

Major clinical studies have recently refuted the idea that estrogen protects women against coronary heart disease (CHD) while ample evidence exculpates androgens for increased CHD risk in men. To investigate the potential contribution of X-chromosome gene(s) to protection from CHD, we examined CHD risk factors in women with Turner's syndrome (TS). TS is characterized by short stature, ovarian dysgenesis, cardiovascular anomalies, and premature CHD. To control for the ovarian failure in TS, we compared glucose and lipid metabolism in lean, young women with TS and age- and body composition–matched women with karyotypically normal premature ovarian failure (POF).

Body mass (BMI) and percent adipose tissue are similar in the two groups, but total cholesterol, LDL cholesterol, and triglycerides are all significantly increased in TS. Moreover, NMR spectroscopy revealed a concentration of smaller, denser HDL and LDL lipid particles in women with TS in contrast to those with karyotypically normal POF. The data show a distinctly atherogenic lipid

TABLE 3.1

Metabolic Parameters in Women with Turner's Syndrome and Premature Ovarian Failure

	Normal Range	TS	POF	P
Age		34±2	34±1	0.9
BMI	20-30 kg/m2	24±1	23±0.5	0.6
Percent Body Fat	20-33%	33±1	32±1	0.3
Total Cholesterol	100-200 mg/dl	208±8	183±5	0.006
LDL	65-129 mg/dl	132±7	109±5	0.007
Triglyceride	10-140 mg/dl	103±9	78±6	0.02
Fasting Insulin	6-27 µU/ml	5.7±0.5	5.5±0.5	0.7

profile in otherwise healthy, nonobese young women with TS (Table 3.1).

Diabetes mellitus (DM) is another major risk factor for CHD. Reports on DM in TS have varied widely, with some studies indicating an increased prevalence up to 40 percent and others finding no increase over normal populations. We have now shown that, while most girls and women with TS have normal fasting glucose and insulin, the glycemic response to a glucose challenge in about 40 percent of such girls and women is dramatically abnormal and consistent with DM and is significantly above the POF control group in *all* women with TS. Interestingly, the glucose intolerance in these young lean women and girls with TS is not explained by insulin resistance but rather by a novel insulin secretory defect. The insulin response to an oral or IV glucose challenge is significantly lower than POF or normal controls in *all* women with TS. It thus appears that the Turner's "metabolic syndrome" is not secondary to obesity or hypogonadism as previously thought. Rather, it is a distinct entity characterized by decreased insulin secretion reminiscent of "transcription factor" mature onset diabetes of the young (MODY) syndromes and is caused by haploinsufficiency for autosomal genes involved in pancreatic development. The implication is that, in TS, haploinsufficiency for unknown X-chromosome gene(s) impairs beta cell function and predisposes to diabetes mellitus.

Our new findings implicating haploinsufficiency for X-chromosome genes in dyslipidemia and diabetes explain the increased risk for CHD in women with TS and may suggest that haploinsufficiency also contributes to the increased risk for CHD among normal XY men as compared with women. The identification of these genes clearly is of great clinical importance. We are using traditional approaches through genotype-phenotype evaluations in subjects with informative X-chromosome deletions or rearrangements and novel bioinformatic strategies to identify functionally interesting X-chromosome sequences likely to escape inactivation.

Early studies reported an increased prevalence of osteoporosis and bone fractures in TS, but these studies relied on areal densitometric measurements, which underestimate bone mineral density (BMD) in small individuals. Furthermore, the studies included women who received estrogen replacement therapy. We have shown that women with TS who have received standard estrogen therapy beginning by age 16 have normal or near normal trabecular BMD as measured by volumetric methods not dependent on bone size. Cortical BMD, however, is selectively reduced in women with TS, independent of estrogen treatment or size. The latter deficit may be attributable to *SHOX* haploinsufficiency, which impairs long bone development. The defect in cortical bone does not, however, appear to lead to increased fragility as there is no excess of fractures in girls or women with TS who have taken estrogen as recommended.

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The role of androgen in protecting the mammary gland

Zhou, Dimitrakakis, Bondy

The normal ovary produces abundant quantities of testosterone in addition to estradiol, but usual hormone replacement treatment (HRT) for ovarian failure consists of estrogen and progesterone for most women with a uterus or of estrogen alone for smaller numbers of hysterectomized women. With such treatment, however, the risk of breast cancer is increased in postmenopausal women, limiting HRT's usefulness. We have proposed that an important role for endogenous androgen in women is to protect the mammary gland from "unopposed" estrogenic stimulation. To evaluate the efficacy of physiologic androgen supplementation in limiting mammary epithelial proliferation induced by estrogen replacement therapy, we employed an ovariectomized rhesus monkey model of menopause. Proliferation increased about four-fold in the estradiol-treated group but did not differ from vehicle-treated control in the estradiol plus testosterone group. As a potential explanation for the mechanism of testosterone's protective effects, we found that testosterone down-regulates estrogen receptor (ER) alpha and up-regulates estrogen receptor beta. Given that the alpha isoform promotes proliferation and MYC expression while the beta isoform does not, the dramatic alteration in ER alpha/beta ratio may be pivotal to testosterone's inhibition of estrogen-induced proliferation. Indeed, we demonstrated a significant reduction in MYC expression in the estrogen/testosterone group. Importantly, we have found that treatment of normally cycling rhesus monkeys with an androgen receptor antagonist increases mammary epithelial proliferation by about 50 percent, indicating that endogenous androgens normally suppress mammary epithelial proliferation. Our observations suggest that endogenous androgens normally limit mammary epithelial proliferation and that androgen supplementation of estrogen therapy may reduce estrogen-induced proliferation and breast cancer risk. These considerations are of crucial importance for young women with ovarian failure who face a long period of HRT as well as for those postmenopausal women who need HRT.

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IGF1 and the brain

Cheng, Wang, Smith, Bondy

The growing brain consumes about 50 percent of the total fuel available to the organism as a whole during early postnatal development, despite the fact that its mass is less than 10 percent of total body mass. How the brain competes so successfully with peripheral tissues for energy and substrates has been unclear. Insulin preferentially enhances fuel use by peripheral tissues, but not by brain. We have shown that endogenous brain IGF1 plays an insulin-like role in promoting neuronal glucose utilization and hence growth during postnatal development. We have also shown that brain growth in *Igf1* null mice falls behind that of normal littermates by almost 40 percent during the postnatal period when brain IGF1 expression is normally most abundant. Over this past year, we have investigated

IGF1's role in neuronal and glial cell generation, survival, growth, and morphogenesis. While neuronal cell numbers are preserved throughout most brain structures in the *Igf1* null brain, we have observed a significant reduction in hippocampal dentate granule cell number. Cell proliferation is normal, but cell death is higher in the *Igf1* null dentate germinal zone, explaining the reduction in dentate size. Neuronal numbers are preserved in the *Igf1* null frontoparietal cortex, but morphometric analysis showed that pyramidal neuron soma size decreases by about 10 percent while Golgi staining showed a significant reduction in pyramidal dendritic length and complexity in *Igf1* null mice. In addition, the density of dendritic spines and presumably synaptic contacts is 16 percent lower in the *Igf1* null brain. We have also found a significant reduction in astrocyte numbers in the *Igf1* null neocortex. The reduction appears to result from reduced proliferation of glial cell precursors in the subependymal zones of the anterior lateral ventricles in the early postnatal period. Taken together, our findings illustrate multifaceted roles for IGF1 in postnatal brain development and explain why individuals with IGF1 gene deletions demonstrate mental retardation in addition to short stature.

We have been interested in factors that regulate brain IGF and IGF receptor expression. The ketogenic diet is a high-fat, low-carbohydrate diet used for treating refractory epilepsy in children. Despite a long history of the diet's clinical use, it is still not entirely clear how the ketogenic diet affects the brain and what mechanism(s) underlie its seizure-suppressive action. Reduction in brain energy supply, e.g., from systemic hypoglycemia or from reduced brain GLUT1 expression, induces seizure activity by impairing the ability of neurons to stabilize membrane potential. We have previously shown that IGF1 is a key regulator of glucose transport and utilization in the developing brain; furthermore, nutritional factors in many different tissues regulate expression of IGF system components. We therefore tested the hypothesis that the ketogenic diet enhances IGF1 activity, thereby improving energy utilization and protecting from seizures, in an animal. We found that the ketogenic diet nearly doubles IGF1 receptor expression in the brains of rats fed a ketogenic diet, providing a possible explanation for the diet's beneficial effects (see Figure 3.1).

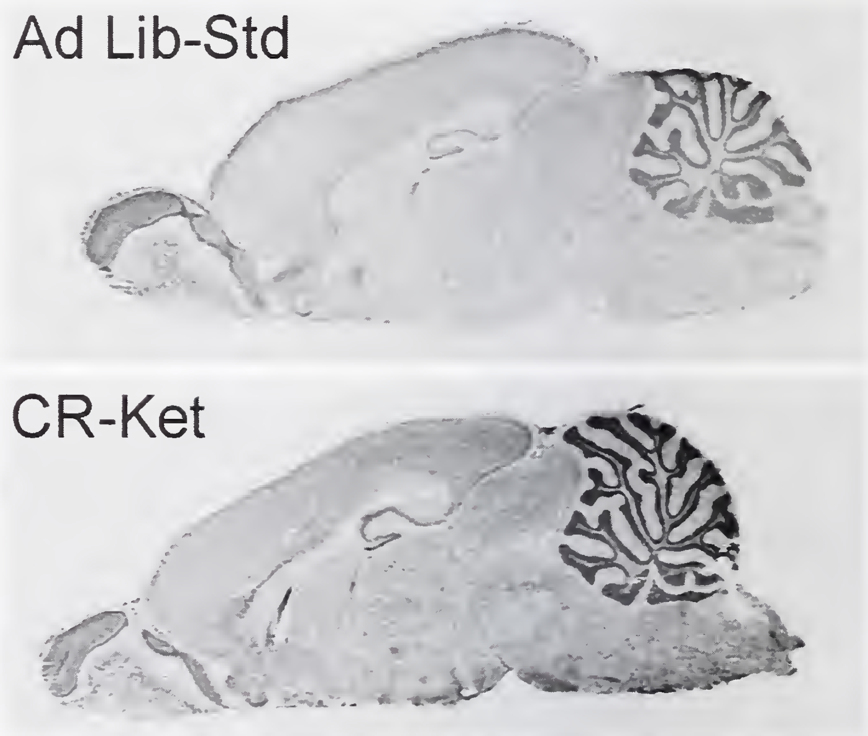

FIGURE 3.1

The ketogenic diet increases brain IGF receptor expression. The figure compares IGF1 receptor mRNA levels in representative film autoradiographs of sagittal brain sections from animals on control diet (upper panel) and ketogenic diet (lower panel). Adapted from Cheng, Kelley, *et al.*, 2003.

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To develop diagnostic tools and therapeutic approaches for menstrual cycle and human reproduction disorders, we investigate genetic, immunological, and molecular aspects of such disorders, using autoimmune premature ovarian failure (POF) as a model condition. POF causes young women to develop amenorrhea and infertility before age 40 and was once considered an irreversible condition similar to normal menopause. We now know that, even though they fail to function normally, follicles remain in the ovaries of 50 percent of patients with POF. In 90 percent of such cases, the mechanism of the ovarian insufficiency remains a mystery. Work in neonatally thymectomized B6A mice, which display autoimmune oophoritis and ovarian failure similar to human autoimmune ovarian failure and are thus a model of autoimmune POF, led us to discover Mater, a novel oocyte protein that serves as a major antigen in ovarian autoimmunity. Using transgenic technology, we also demonstrated that Mater is essential for normal female fertility. In addition, we have been investigating clinical POF by recruiting patients to research protocols that are designed to gain insight into the mechanisms of ovarian follicle dysfunction and to develop appropriate treatments for patients with 46,XX spontaneous POF.

Premature ovarian failure, autoimmune oophoritis, and steroidogenic cell autoantibodies

Anasti, Vanderhoof, Nelson; in collaboration with Bakalov, Bondy, Calis

Evidence is accumulating to suggest that autoimmune oophoritis and adrenal autoimmunity may represent a continuum of one pathophysiologic process. We have been conducting an IRB-approved protocol investigating autoimmune oophoritis as a mechanism causing 46,XX spontaneous POF. We are studying the accuracy of steroidogenic cell antibodies in predicting the presence of autoimmune oophoritis and collating results of the antibody test with a gold standard: autoimmune oophoritis confirmed by ovarian biopsy. Given that ascertainment bias has created uncertainty about the true prevalence of autoimmune lymphocytic oophoritis as a mechanism of POF, we are studying the prevalence of steroidogenic cell autoantibodies in young women with 46,XX spontaneous POF who meet two primary criteria: (1) infertility and amenorrhea as a primary concern and (2) otherwise general good health.

It is well established that patients with spontaneous POF are at increased risk of developing autoimmune adrenal insufficiency, a potentially fatal disorder. Adrenal insufficiency has an insidious onset, and patients with the condition often experience a delay in diagnosis. There has been ongoing controversy regarding the best strategy to detect adrenal insufficiency at an early stage, and we recently studied the effectiveness of antisteroid cell antibody detection by indirect immunofluorescence as a screening test. We comprehensively evaluated the adrenal axis of 123 women with spontaneous POF and diagnosed asymptomatic adrenal insufficiency in four women who tested positive for adrenal antibodies. A positive adrenal antibody test was highly associated with adrenal insufficiency while a negative test was associated with normal adrenal function in all cases; the cortisol response during a standard adrenocorticotrophic hormone (ACTH) stimulation test gave two false positive results. Thus, measuring adrenal antibodies is an effective screening method with which to detect asymptomatic autoimmune adrenal insufficiency in young women with spontaneous POF, whereas the standard ACTH stimulation test should be reserved to confirm adrenal insufficiency in women who test positive for adrenal antibodies or in women with signs and symptoms of adrenal insufficiency.

Bakalov VK, Vanderhoof VH, Bondy CA, Nelson LM. Adrenal antibodies detect asymptomatic autoimmune adrenal insufficiency in young women with spontaneous premature ovarian failure. *Human Reprod* 2002;17:2096-2100.

Antigenic role of MATER in autoimmune premature ovarian failure

Vanevski, Soto, Tong, Nelson

Although autoantibodies develop in most autoimmune diseases, the existence of specific ovarian antigens involved in human autoimmune POF has yet to be confirmed. Autoimmune POF causes young women to develop amenorrhea, menopausal symptoms, and infertility. We have been developing a serum marker that will identify women who have POF due to autoimmunity and a treatment that will restore fertility. We have demonstrated that mice with autoimmune oophoritis develop antibodies against MATER. Experiments are under way to test the antigenic role of MATER in the development of autoimmune POF in mice and in women.

We recently defined the human homolog of the mouse *Mater* and have initiated efforts to produce human recombinant MATER and to develop an assay to determine if some women with 46,XX spontaneous POF exhibit autoantibodies to this oocyte-specific protein. We previously reported that the maternal effect gene *Mater* is required for early embryonic development in mice. We cloned human *MATER* cDNA by PCR techniques and found that the human MATER gene spans about 63 kbp at chromosome 19 and comprises 15 exons and 14 introns. Expression of its mRNA is restricted to the oocytes. Human *MATER* cDNA shows an open reading frame encoding a polypeptide chain of 1,200 residues with a predicted molecular mass of 134,236 Da, and MATER protein was detected in human oocytes. The human and mouse cDNA share 67 percent homology while their deduced polypeptide chains have 53 percent amino acid identity. In addition, their protein structures have a number of similar features. Characterization of human *MATER* and its protein provides a basis for us to investigate their clinical implications in autoimmune POF and infertility in women.

During the last year, we demonstrated the unreliability of a commercially available ovarian antibody test that uses cynomologous monkey ovary as substrate in an indirect immunofluorescence procedure. Some clinicians use this test to make treatment decisions, but, to our knowledge, the test had never been validated. We performed the test in a blinded manner in young women with 46,XX spontaneous POF, in control women with regular menstrual cycles (matched for age, race, and parity), and in control men (matched for age and race). We also compared the frequency of other autoantibodies associated with ovarian autoimmunity and found that women with POF were more likely to exhibit thyroid peroxidase and parietal cell antibodies. Unexpectedly, use of the commercially available test revealed that nearly one-third of normal control women had ovarian antibodies. One half of young women with POF were found to have antibodies, but the frequency of a positive test was not significantly different from that in normal women. None of 26 men was found to have ovarian antibodies. We were unable to improve the specificity of the test and thus conclude that the method has poor specificity and is not a reliable tool for the diagnosis of autoimmune ovarian failure. Our findings underscore the need to demonstrate the specificity of any ovarian antibody test before it is used clinically.

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Low follicle number as a mechanism of 46,XX spontaneous premature ovarian failure

Tong, Udofa, Emeche, Nelson

We previously demonstrated that the mere existence of a low number of follicles creates an indirect mechanism causing follicle dysfunction in young women with 46,XX spontaneous POF. We demonstrated histologically that the follicle dysfunction is mediated by inappropriate luteinization and that the mechanism relates to inadequate negative feedback on gonadotropins due to a low follicle cohort size. For this reason, we are looking for gene mutations that might cause germ cell deficiency and low cohort size as a mechanism of POF. Given that mice with mutations in the c-kit receptor have a reduced number of germ cells, we recently tested the hypothesis that such mutations are a cause of reduced germ cell number. The c-kit system, including c-kit receptor and its ligand, is known to play important regulatory roles in ovarian folliculogenesis and oogenesis. However, we found that mutations in the coding region of the kit receptor gene are uncommon in women with POF. In ongoing work, we are evaluating the kit ligand gene and other candidate genes in which mutations are known to cause ovarian follicle deficiency in mice.

Nelson LM, Bakalov VK. Mechanisms of follicle dysfunction in 46,XX spontaneous premature ovarian failure. *Endocrinol Metab Clin North Am* 2003;32:613-637.

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Needs of young women with 46,XX spontaneous premature ovarian failure

Groff, Spitalnik, Pastor, Vanderhoof, Nelson; in collaboration with Calis, Smith

We are conducting clinical research to define more fully other needs of young women with 46,XX spontaneous POF. In B6A mice, neonatal thymectomy induces lacrimal gland autoimmunity as well as autoimmune ovarian failure and provides the basis for an animal model of Sjögren's syndrome. To test the hypothesis that a similar association may be present in a subset of women with 46,XX spontaneous POF, we are collaborating with investigators at the National Eye Institute. We found an association between ocular surface disease and POF that has not been reported before. Keratoconjunctivitis sicca is defined as at least one symptom of dry eye occurring often or present all of the time. There are two major mechanisms of keratoconjunctivitis defined on the basis of either aqueous tear deficiency or evaporative tear deficiency. A decreased volume of tear production characterizes aqueous tear deficiency and may be the result of autoimmunity directed against the lacrimal glands. A qualitative disturbance in the tear film with resultant instability, leading to increased evaporation and dryness of the ocular surface, characterizes evaporative tear deficiency and is most frequently the result of meibomian gland disease. Meibomian gland dysfunction leads to decreased lipid production, which results in tear film instability. Interestingly, androgens, which may be deficient in women with POF, appear to play an important role in supporting normal meibomian gland function. Compared with age-matched control women, we found that young women with 46,XX spontaneous POF have an increased prevalence and severity of both signs and symptoms of ocular surface disease. Given that not all patients had dry eye, it is possible that the dry eye phenotype may signal a particular mechanism of POF, such as autoimmunity. It is also possible that endocrine factors, such as the androgen deficiency associated with POF, might explain the association. Additional studies are planned to characterize this pathology further and determine its etiology.

We also recently completed an investigation of the experiences of young women with spontaneous POF with regard to their initial presenting symptom, promptness of diagnosis, and patient education.

We asked 50 patients previously diagnosed with spontaneous POF to participate in a structured interview. Disturbance in menstrual pattern was the most common initial symptom. Over half of the women who presented with this complaint reported visiting three different clinicians' offices before laboratory testing was performed to determine the diagnosis. For 25 percent of the women, it took more than five years for the diagnosis of POF to be established. A majority of the women were unsatisfied with the manner in which they had been informed of the diagnosis and with the amount of information they had received about their condition. Our findings demonstrate that women with spontaneous POF perceive a need for more aggressive evaluation of secondary amenorrhea and oligomenorrhea. Menstrual irregularity can be a sign of ovarian insufficiency, and the associated estrogen deficiency is a well-established risk factor for osteoporosis. Indeed, in an earlier study, we found that two-thirds of young women with spontaneous POF had osteopenia of the femoral neck.

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Smith JA, Vitale S, Reed GF, Grieshaber SA, Goodman LA, Vanderhoof VH, Calis KA, Nelson LM. Dry eye signs and symptoms in women with premature ovarian failure. *Arch Ophthalmol*; in press.

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MOLECULAR GENETICS OF ADRENOCORTICAL TUMORS AND RELATED DISORDERS

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The goal of our work is to understand the genetic and molecular mechanisms leading to disorders that affect the adrenal cortex, with emphasis on those that are developmental, hereditary, and associated with adrenal hypoplasia or hyperplasia, multiple tumors, and abnormalities in other endocrine glands (especially the pituitary gland and, to a lesser extent, the thyroid gland). We have studied congenital adrenal hypoplasia caused by triple A syndrome and multiple endocrine deficiencies, familial hyperaldosteronism, adrenocortical and thyroid cancer, and pituitary tumors; multiple endocrine neoplasia (MEN) syndromes affecting the pituitary, thyroid, and adrenal glands; and Carney complex (CNC), an autosomal dominant disease. CNC is a MEN syndrome affecting the pituitary, adrenal cortex, thyroid, and the gonads, and is associated with a variety of other tumors, including myxomas and schwannomas, and skin pigmentation defects (lentigines, café-au-lait spots, and nevi). We have identified the regulatory subunit type 1-alpha (RIalpha) of protein kinase A (PKA), which is coded by the *PRKAR1A* gene as the gene responsible for most CNC patients. Thus, a significant part of our work is now focused on PKA-stimulated signaling pathways, PKA effects on tumor suppression and/or development, the cell cycle, and chromosomal stability. These projects are facilitated by *Prkar1a*-specific animal models.

Carney complex genetics

Bauer, Bei, Bourdeau, Griffin, Matyakhina, Stergiopoulos, Stratakis; in collaboration with Bertherat, Carney, Kirschner

Families with Carney complex and related syndromes have been collected from a number of collaborating institutions worldwide. Loci harboring genes for CNC on chromosomes 2 (2p16) and 17 (17q22-24) were identified through genetic linkage analysis; other possible loci for this genetically heterogeneous condition are currently under investigation. Using molecular cytogenetic techniques, we are investigating the participation of these genomic loci in the expression of the disease. We have constructed a comprehensive genetic and physical map of the 2p16 chromosomal region for the cloning of the CNC-associated sequences from the region. Studies in cultured primary tumor cell lines (established from our patients) identified a region of genomic amplification in CNC tumors in the center of the map. The gene responsible for CNC in most cases of the disease, *PRKAR1A* on 17q22-24, appears to undergo loss of heterozygosity in at least some CNC tumors. *PRKAR1A* is also the main regulatory subunit of protein kinase A (PKA), a central signaling pathway for many cellular functions and hormonal responses. Patients with CNC are now being studied for genotype-phenotype correlations, which are expected to shed light on the complex biochemical and molecular pathways regulated by *PRKAR1A* and PKA (see Figure 3.2).

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Matyakhina L, Pack S, Kirschner LS, Pak E, Mannan P, Jaikumar J, Taymans SE, Sandrini F, Carney JA, Stratakis CA. Chromosome 2 (2p16) abnormalities in Carney complex tumours. *J Med Genet* 2003;40: 268-277.

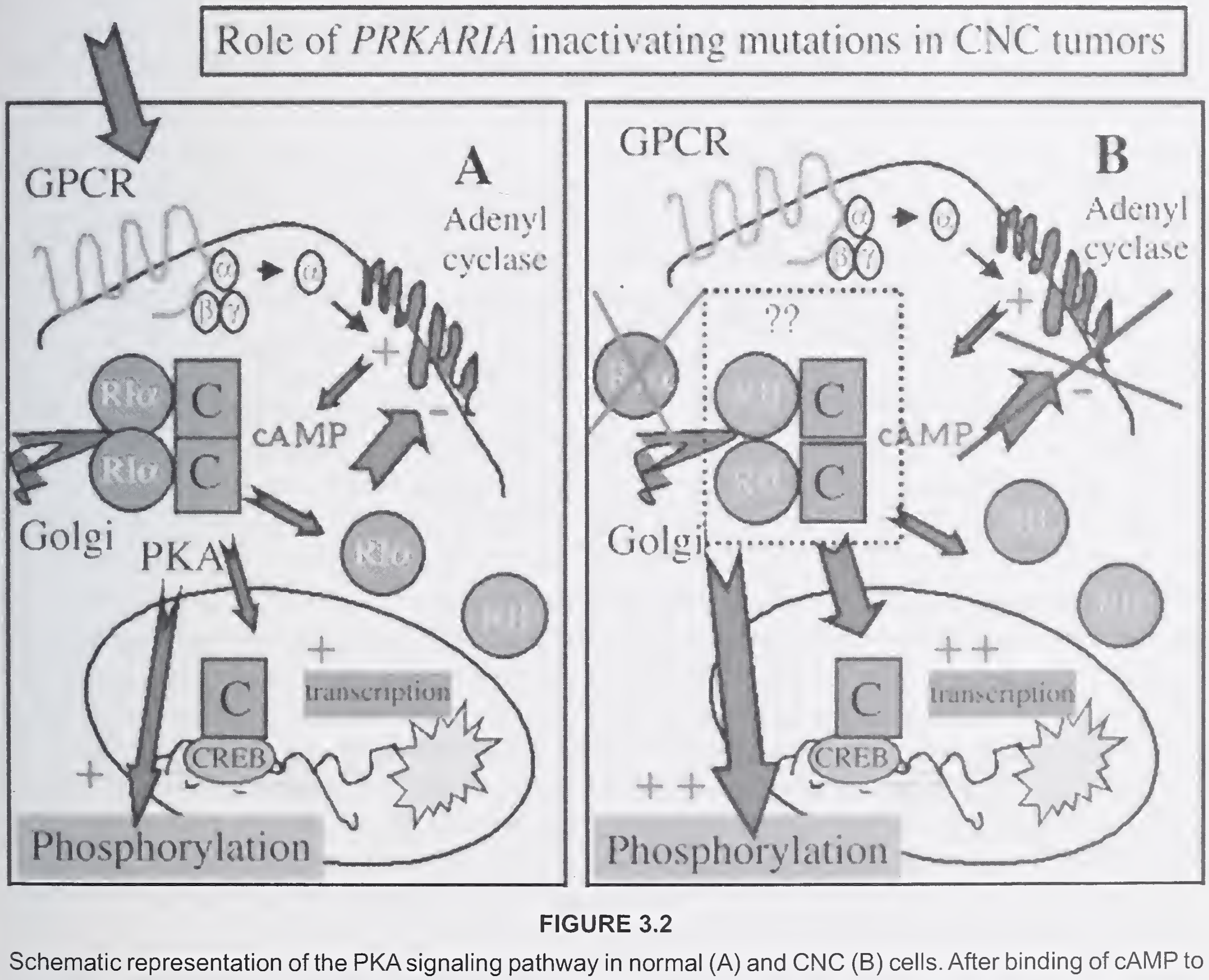

FIGURE 3.2

Schematic representation of the PKA signaling pathway in normal (A) and CNC (B) cells. After binding of cAMP to the homodimer of regulatory subunits, PKA is activated. Catalytic subunits are released following conformational changes of the regulatory subunits; phosphorylation of cytoplasmatic targets ensues, often resulting in “cross-talking” with other intracellular signaling pathways (such as the MAPK). In the nucleus, PKA catalytic subunits phosphorylate CREB, resulting in activation of DNA transcription of cAMP-responsive element (CRE)–containing genes. In a *PRKAR1A* mutant cell, with no or an ineffective regulatory subunit type 1A, there is a compensating excess of the other regulatory subunits, which may also lead to an increased availability of free catalytic subunits and perhaps decreased inhibition of phosphodiesterases and/or other feedback molecules. The net effect of all these changes in CNC cells is an increase in DNA transcription and/or activation of other pathways leading to abnormal growth and proliferation.

Sandrini F, Stratakis CA. Clinical and molecular genetics of Carney complex. *Mol Genet Metab* 2003;78:83-92.

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PRKAR1A effects on protein kinase A activity and endocrine tumor development

Stergiopoulos, Matyakhina, Robinson-White, Stratakis; in collaboration with Bertherat, Cho-Chung

The functional consequences of *PRKAR1A* mutations are under investigation in cell lines established from CNC patients and their tumors. We measure both cAMP and PKA activity in these cell lines, along with the expression of the other subunits of the PKA tetramer. We have established stable transfectants of antisense *PRKAR1A* constructs in commercially available mouse endocrine and other cell lines and have studied the effects of *PRKAR1A* silencing on the cells' growth, differentiation, and proliferation. We hypothesize that the tumorigenicity of *PRKAR1A*-inactivating mutations relies on the switch from type-I PKA (based almost exclusively on endocrine cells) to type-II PKA activity (see Figure 3.2); cell lines with an antisense *PRKAR1A* construct are believed to be a representative model of the *in vivo* situation in CNC patients. In addition to the above, we are seeking mutations of the *PRKAR1A* gene in sporadic endocrine and nonendocrine tumors (thyroid adenomas and carcinomas, adrenocortical adenomas and carcinomas, ovarian carcinomas, melanomas and other benign and malignant pigmented lesions, and heart myxomas) to establish further the gene's role as a general tumor suppressor; several investigators within the NIH and around the world provide the specimens.

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Prkar1a$^{+/-}$ animal model and tissue-specific *Prkar1a* analysis

Bauer, Griffin, Stergiopoulos, Claflin, Meoli, Weinberg, Stratakis; in collaboration with Kirschner, Westphal

The *Prkar1a*-knock-out (KO) (-/-) mouse, an animal model created several years ago by S. McKnight (University of Washington, Seattle), dies early in embryonic development because of heart and central nervous system abnormalities. Since the discovery of *PRKAR1A*'s involvement in CNC, Lawrence Kirschner in our laboratory, in collaboration with Heiner Westphal (NICHD) developed a *Prkar1a* KO floxed by a lox-P system to generate, first, a novel *Prkar1a*$^{+/-}$ and, second, knockouts of the *Prkar1a* gene in a tissue-specific manner after crossing the new mouse model with mice that express the *cre* protein in the respective endocrine tissues (adrenal cortex, anterior lobe of the pituitary, and the thyroid gland).

Kirschner LS, Towns WH, Williams-Simons LM, Westphal H, Stratakis CA. Generation of new mouse lines expressing cre recombinase in endocrine tissues. *Endocrine Society Philadelphia,* Abstract 2003;OR-24-6.

Antisense *Prkar1a* transgenic mouse model

Griffin, Stergiopoulos, Claflin, Meoli, Weinberg, Stratakis; in collaboration with Bornstein, Kirschner

Given that the *Prkar1a*$^{+/-}$ model was not known to develop tumors, we hypothesized that a different system would have a better chance of reproducing the human disease caused by a haploinsufficient *PRKAR1A* gene. Thus, in addition to the model described above, we created a transgenic (TG) mouse carrying an antisense (AS) transgene for exon 2 of the mouse *Prkar1a* gene (X2AS) under the control of a regulatable promoter. We have now completed our examination of cAMP-stimulated kinase activity, pathology, and immunohistochemistry of the TG mice at six to eight months of age. Mice expressing the X2AS construct displayed normal reproductive behavior but showed marked differences in reproductive efficiency (presumably due to *in utero* death of those expressing high levels of X2AS). As is seen in human CNC tumors, tissues from mice with the X2AS transgene showed higher cAMP-stimulated kinase activity. Although no tumors were found in endocrine tissues at age six to eight months, the mice exhibited some CNC-compatible histologic changes. Continuing observation of the animals and further studies may provide insight into the mechanisms leading to cAMP-related abnormal growth and proliferation (see Figure 3.2) in CNC.

Griffin KJ, Kirschner LS, Stergiopoulos SG, Lenherr SM, Claflin ES, Matyakhina L, Bauer AJ, Carney AJ, Stratakis CA. A transgenic mouse model expressing an antisense construct to PRKAR1A: a model for Carney complex. *Endocrine Society Philadelphia,* Abstract 2003;P2-54.

PRKAR1A, the cell cycle, chromosomal stability, mitogen-activated protein kinases (MAPK), and other signaling pathways

Matyakhina, Meoli, Shiferaw, Robinson-White, Stratakis; in collaboration with Bornstein, Grimberg, Papdopoulos

This work aims to identify *PRKAR1A*-interacting mitogenic and other growth signaling pathways in cell lines expressing *PRKAR1A* constructs and/or mutations (see Figure 3.2). In addition, by using classic and molecular cytogenetics, including fluorescent *in situ* hybridization (FISH), spectral karyotyping (SKY), and comparative genomic hybridization (CGH), we are studying chromosomal stability in both human and mouse cell lines in which *PRKAR1A* has been inactivated. Genes implicated in cyclic nucleotide–dependent signaling have long been considered likely candidates for endocrine tumorigenesis. Somatic activating mutations in a number of G protein–coupled receptors (GPCRs) and in the gene encoding a subunit of the stimulatory G protein (*GNAS1*) lead to increased cAMP production and are responsible for a number of endocrine tumors of various types. To date, however, there is no convincing evidence that GNAS1 or GPCR activation, in the absence of additional genetic abnormalities, is involved in cancer. Individuals with McCune-Albright syndrome (MAS), a disease with similarities to CNC, who bear somatic GNAS1 mutations in their endocrine glands, may be predisposed to develop some cancers. Activation of additional pathways and/or other changes appear to be required for the *in vitro* transformation of 3T3 or FRTL5 cells by a constitutively active GPCR transgene or in other settings of increased cAMP signaling that lead to malignant transformation. We recently showed that the MAPK ERK1/2 pathway is activated in haploinsufficient *PRKAR1A* cell lines. Other genes that regulate PKA function and increase cAMP-dependent proliferation and related signals may be altered in the process of endocrine tumorigenesis initiated by a mutant *PRKAR1A*, a gene with important functions in the cell cycle and chromosomal stability. In collaboration with Vassilios Papadopoulos, we have identified proteins that are directly bound to *PRKAR1A* (RIalpha) and regulate its function, including the novel protein PAP7. Compartmentalization of PKA function is thought to be mediated by anchoring proteins; almost all the PKA-anchoring proteins known to date, however, are bound to type II PKA. PAP7 may be the first PKA type-I–specific anchoring protein.

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Robinson-White A, Stratakis CA. Protein kinase A signaling: "Cross-talk" with other pathways in endocrine cells. *Ann N Y Acad Sci* 2002;968:256-270.

Genetic investigations on other adrenocortical diseases and tumors

Bei, Bourdeau, Farrell, Stergiopoulos, Stratakis; in collaboration with Bertherat, Bossis, Carney, Chan, Hammer, Lacroix, Libutti, Stowasser, Torpy, Voutetakis

This work aims at (1) using general and pathway-specific microarrays on a variety of adrenocortical tumors, including single adenomas and massive macronodular adrenocortical disease (MMAD) (see Figures 3.3 and 3.4), to identify genes with important functions in adrenal oncogenetics; (2) examining specific candidate genes (such as *TP53* and other tumor suppressors and oncogenes) for their roles in adrenocortical tumors and development; and (3) identifying by positional cloning additional genes with a role in inherited adrenocortical and related diseases. Most of the work is performed collaboratively.

Carney JA, Stratakis CA. Familial paraganglioma and gastric stromal sarcoma: a new syndrome distinct from the Carney triad. *Am J Med Genet* 2002;108:132-139.

Genetic investigations on pituitary tumors, other endocrine neoplasias, and related syndromes

Bei, Bourdeau, Bauer, Farrell, Stergiopoulos, Stathatos, Stratakis; in collaboration with Francis, Marx, Ringel

Additional work in our laboratory focuses on the genetics of CNC- and adrenal-related endocrine tumors, including childhood adrenocortical cancer and thyroid and pituitary tumors. As part of this work, we have described novel genetic abnormalities in thyroid tumors. In addition, the genetic defects in patients with CNC-related syndromes (the lentigenoses, i.e., Peutz-Jeghers syndrome and others) are undergoing identification. The work is performed collaboratively with a number of other investigators at the NIH and elsewhere.

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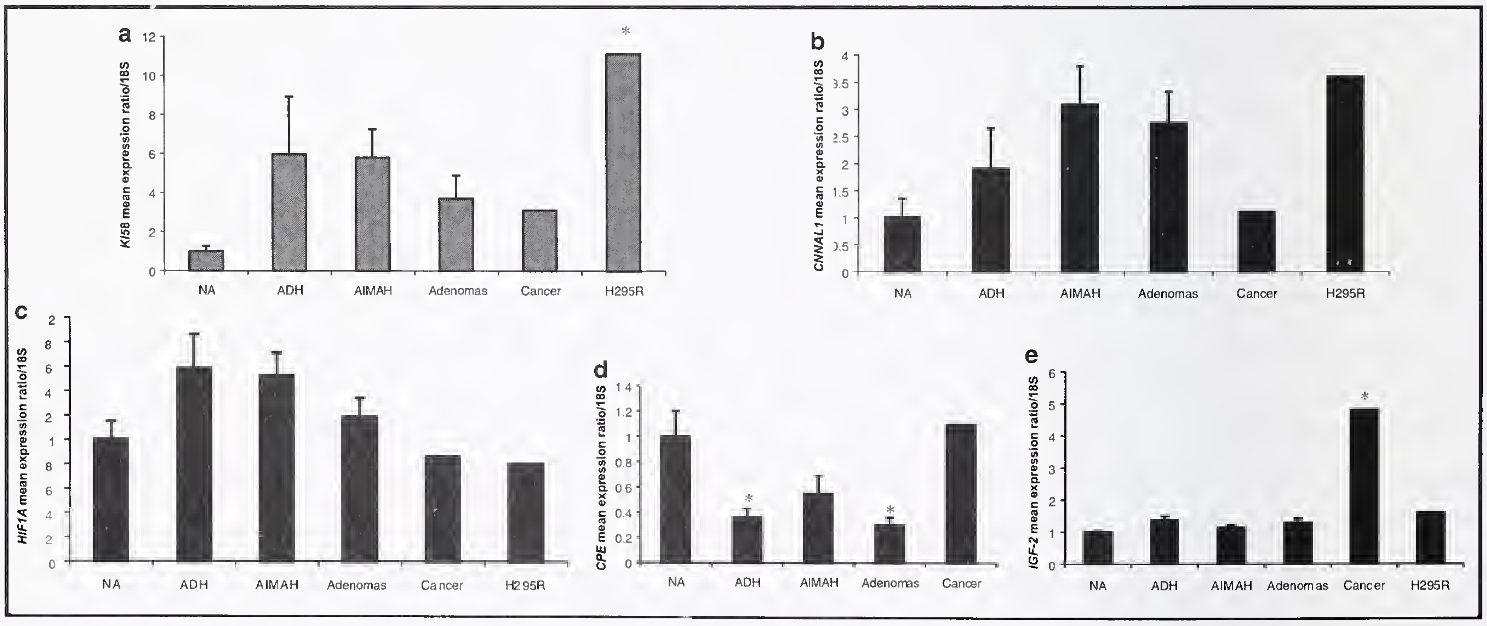

FIGURE 3.4

Mean expression ratio of *KIF5B (a), CNNAL1 (b), HIF1 A (c), CPE (d)* and *IGF2 (e)* by multiplex RT-PCR in normal adrenal glands (NA), ACTH-dependent adrenal hyperplasia (ADH), ACTH-independent macronodular adrenal hyperplasia (AIMAH), adrenal adenomas, cancer, and the H295R human adrenocortical carcinoma cell line. Expression of *IGF2* was not significantly altered overall in AIMAH compared with normal adrenal (NA), whereas *IGF2* was clearly highly expressed in both cancer and the H295R cell line; there was, however, significant heterogeneity in the data set. Errors bars represent standard error of the mean; *significance at $P < 0.05$.

Clinical investigations in the diagnosis and treatment of adrenal and pituitary tumors

Griffin, Bourdeau, Batista, Stratakis; in collaboration with Keil, Patronas

Patients with adrenal tumors and other types of Cushing's syndrome (and occasionally other pituitary tumors) come to the NIH Clinical Center for diagnosis and treatment. Ongoing investigations study (1) the prevalence of ectopic hormone receptor expression in adrenal adenomas and MMAD; (2) the diagnostic use of high-sensitivity magnetic resonance imaging for the earlier detection of pituitary tumors; and (3) the diagnosis, management, and postoperative care of children with Cushing's disease and other pituitary tumors.

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PHYSIOLOGY, PSYCHOLOGY, AND GENETICS OF OBESITY

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The prevalence of overweight and obesity in children and adults has tripled during the past 30 years. The alarming rise in body weight has likely occurred because the current environment affords easy access to calorie-dense foods and requires less voluntary energy expenditure. However, this environment leads to obesity only in those individuals whose body weight regulatory systems are not able to control body adiposity with sufficient precision in our high-calorie/low-activity environment, which suggests that some subgroups in the United States have a uniquely high susceptibility to weight gain under prevailing environmental conditions. Our research is directed at increasing our understanding of the environmental, metabolic, and behavioral factors involved in determining body weight regulation and body composition during childhood, with a special emphasis on minority populations. The ongoing research program prospectively evaluates risk factors for the development of obesity and its complications in children, studies the effects of medications on body weight and obesity-related comorbid conditions in children, and seeks the genetic and environmental factors important for the markedly greater incidence of obesity and its comorbid conditions in some U.S. minority populations.

Molecular studies of factors important for childhood body weight regulation

Feng, Adler-Wailes, Elberg, Carr, Liu, Yaniv, J. Yanovski; in collaboration with Warden

To attempt to identify gene variants affecting body composition with different frequency in African American and Caucasian children, we are using classical association studies to investigate polymorphisms in genes involved in the leptin signaling pathway. Genes currently under study include those encoding proopiomelanocortin (POMC), POMC processing enzymes, the melanocortin receptors 3, 4, and 5, and neuropeptide Y and its receptors. In addition, we have studied genes important for energy expenditure, such as those encoding mitochondrial uncoupling proteins (UCP), and genes potentially involved in cortisol metabolism that may affect intra-abdominal adipose tissue, such as that encoding 11-beta-hydroxysteroid dehydrogenase. We have found that a 45 base pair insertion polymorphism in the eighth exon of UCP-2 is associated with excess body weight in African American, Asian, and Caucasian children and that an intronic polymorphism in the 11-beta-hydroxysteroid dehydrogenase gene is associated with numerous features of the metabolic syndrome in children. We have also identified novel polymorphisms in the POMC sequence that are more prevalent in African American than Caucasian children but do not appear to play a role in the greater body adiposity of African American children. We are currently studying a variant melanocortin 3 receptor gene that is associated with adiposity in a large cohort of children and that appears to have functional significance for melanocortin 3 receptor signal transduction. Ongoing studies attempt to understand the mechanisms by which the sequence alterations may affect body weight.

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Yaniv LG, Feng NP, Sebring NG, Hochberg Z, Yanovski JA. Associations between a polymorphism in the 11 beta hydroxysteroid dehydrogenase type I gene and body composition. *Int J Obes* 2003;27:983-986.

Physiology, metabolism, and psychology of childhood body weight regulation

Uwaifo, McDuffie, Parikh, Elberg, Fallon, Morgan, Tanofsky-Kraff, Cohen, J. Yanovski; in collaboration with Keil, Schoeller, Warden, S. Yanovski, Young-Hyman

Because the amount of visceral fat in Caucasians is highly associated with the complications of obesity, we have studied the distribution of adipose tissue and assessed insulin resistance and body composition in African American and Caucasian children. We have found less visceral abdominal adipose tissue in nonobese and obese African American children than Caucasian children but considerably greater insulin resistance in African American children. In recent studies, we found the hyperglycemic clamp procedure to be highly correlated with the more technically demanding euglycemic, hyperinsulinemic clamp study in children and therefore suitable for assessing children's insulin sensitivity. African American children were then found to have greater insulin secretion, a greater prevalence of acanthosis nigricans, and lower insulin sensitivity than Caucasian children of similar body composition. The results imply that the relationship between visceral fat and the complications of obesity may differ in African Americans and Caucasians. The susceptibility to weight gain in African Americans may also result from differences in metabolic efficiency. Thus, we have also found that resting energy expenditure is approximately 90 kcal/d less in African American children than in Caucasian normal weight and overweight boys and girls. Our studies suggest that the differences are not explained by differences in levels of the hormone leptin. We have also studied the effects of adiposity on skeletal maturation in African American and Caucasian children, finding that a substantial portion of the advancement of sexual and skeletal maturation found in African American children may be explained by their greater adiposity.

In two ongoing protocols designed to determine the factors most important for the development of complications of obesity in African American and Caucasian youth, we are studying normal weight African American and Caucasian children and adolescents, African American and Caucasian children who are already obese, and the nonobese African American and Caucasian children of obese parents. First, we are examining body composition, metabolic rate, insulin sensitivity, glucose disposal, and genetic factors believed to regulate metabolic rate, such as the uncoupling proteins, leptin and its receptor, melanocortin receptors, and the beta-3 adrenergic receptor. Second, we have recently found that children who report binge-eating episodes during childhood have greater adiposity than those not reporting such episodes. In a longitudinal study from childhood into adulthood, we are defining the psychological and behavioral factors, such as propensity to engage in binge-eating behavior, that contribute to obesity. We hypothesize that differences in these factors will predict the development of obesity in the populations studied and may be of great importance in developing rational approaches for the prevention and treatment of obesity in the diverse U.S. population.

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Treatment of children and adolescents with comorbid conditions associated with obesity

McDuffie, Uwaifo, Parikh, Freedman, Semega-Janneh, Fallon, Tanofsky-Kraff, Cohen, J. Yanovski; in collaboration with Calis, Drinkard, Krakoff, Reynolds, Riggs, Rolls, Salaita, Sebring, Young-Hyman

Given the rapid increase in the prevalence of obesity, the development of treatments for obesity in childhood is urgently needed. In two ongoing clinical protocols, we are studying novel approaches to the control of body weight in children. We have completed a pilot study demonstrating that severely overweight adolescents can lose weight when enrolled in a comprehensive weight management program that includes the novel gastrointestinal lipase inhibitor orlistat as an adjunct to a behavior modification program. We have also found evidence that one mechanism through which orlistat may affect body weight is by changing the hedonic value of dietary fat. An ongoing placebo-controlled randomized trial will determine whether the use of orlistat improves the weight loss of African American and Caucasian children and adolescents with obesity-related comorbidities. The second study examines the mechanism by which another novel weight loss agent, metformin, may affect the body weight of younger children with hyperinsulinemia.

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Environmental factors affecting adult weight gain

Parikh, Edelman, J. Yanovski; in collaboration with Calis, S. Yanovski

We are also interested in assessing the impact of the environment on body weight gain. To determine the role of seasonal variation in weight change, we prospectively studied 200 U.S. adults, following them longitudinally. We found that the only time during which weight changes significantly is in the fall-winter holiday season between Thanksgiving and New Year's Day. Such studies have implications for individuals attempting to control their weight. We are also interested in the role played by dietary calcium in determining body weight change. A new protocol attempts to examine the impact of dietary calcium supplementation on body weight in a randomized, controlled, trial of 340 adults.

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PHOSPHOINOSITIDE-CALCIUM SIGNALING IN CELL REGULATION

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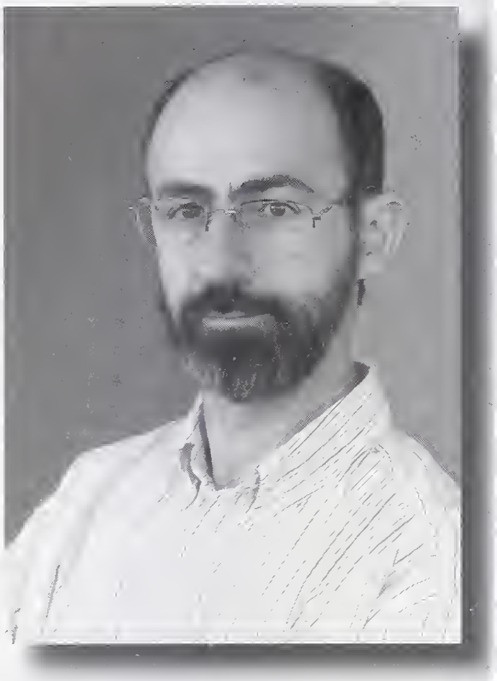

We investigate signal transduction pathways that mediate the actions of hormones and growth factors in mammalian cells, with special emphasis on the role of phosphoinositide-derived messengers. While phosphoinositides constitute a small fraction of the cellular phospholipids, they play a critical role in the regulation of many (if not all) signaling protein complexes that assemble on the surface of cellular membranes. Phosphoinositides regulate protein kinases, GTP-binding proteins, and membrane transporters including ion channels, thereby controlling cellular processes such as proliferation, apoptosis, and metabolism. Our group focuses on the phosphatidylinositol 4-kinases (PI4Ks) that catalyze the first committed step in phosphoinositide synthesis. Current studies are aimed at (1) understanding the function and regulation of several phosphatidylinositol (PI) 4-kinases in the control of the synthesis of hormone-sensitive phosphoinositide pools; (2) characterizing the structural features that determine the catalytic specificity and inhibitor sensitivity of PI 3- and PI 4-kinases; (3) defining the molecular basis of protein-phosphoinositide interactions via the pleckstrin homology and other domains of selected regulatory proteins; (4) developing tools to analyze inositol lipid dynamics in live cells; and (5) determining the importance of the lipid-protein interactions in the activation of cellular responses by G protein–coupled receptors and receptor tyrosine kinases.

Inhibition of PI(3,4,5)P_3-mediated cellular responses by pleckstrin homology domains

Várnai, Bondeva, A. Balla, Barshishat, T. Balla*

Pleckstrin-homology (PH) domains are protein modules of about 150 amino acids with a characteristic fold; they have attracted great interest because of their ability to bind to phosphoinositides. PH domains are present in a large variety of signaling molecules, including tyrosine- or serine-threonine kinases, guanine nucleotide exchange factors, GTPase-activating proteins, phospholipases, and a number of adaptor proteins. Given that several types of regulatory molecules possess PH domains that specifically bind to the same phosphoinositide [i.e., PI(3,4,5)P_3], we wanted to investigate whether PH domains originating from various regulatory proteins and displaying identical lipid-binding specificity would interfere with PI(3,4,5)P_3-regulated cellular responses in a similar manner. Therefore, several PH domains with the ability to bind to phosphatidylinositol 3,4,5,-trisphosphate [PI(3,4,5)P_3] were expressed in NIH 3T3 and COS-7 cells as GFP fusion proteins to determine their effects on various cellular responses known to be activated by PI(3,4,5)P_3. All these proteins, namely, the PH domains of Grp1, Akt, ARNO, and Btk, were found to show PDGF-stimulated and wortmannin-sensitive translocation from the cytosol to the plasma membrane, indicating their ability to recognize PI(3,4,5)P_3. Remarkably, while overexpressed AktPH-GFP and BtkPH-GFP were quite potent in antagonizing the PI(3,4,5)P_3-mediated activation of Akt, overexpression of the other PH domains showed no such inhibitory effect. In contrast, expression of the PH domains of Grp1 and ARNO, but not those of Akt or Btk, inhibited the attachment of freshly seeded cells to culture dishes. Activation of PLCgamma by EGF was also attenuated by the PH domains of Grp1, ARNO, and Akt but was significantly enhanced by the Btk PH domain. Followed over several days, the kinetics of expression of the various GFP-fused PH domains clearly showed a lipid-binding–dependent self-elimination of the PH-domain of Akt, consistent with its interference with the anti-apoptotic Akt signaling pathway. Moreover, NIH 3T3 cells expressing the various PH domains showed characteristic morphological changes: greatly elongated extensions in response to Grp1-PH and ARNO-PH and a larger foot-print size in response to the Akt PH. The data suggest that interaction with and sequestration of PI(3,4,5)P_3 is not the sole mechanism by which PH domains interact with cellular membranes and that PH domains are likely to possess structural features that allow their more specific participation in the assembly and function of specific signaling pathways.

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Characterization of the pleckstrin homology domain of phospholipase C-delta4

Várnai, A. Balla, T. Balla; in collaboration with Jalink, Lee, Rhee

Some of the most important regulators of phosphoinositide levels are the phospholipase C (PLC) enzymes that hydrolyze phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]. Of the various isoforms of PLCs, the delta forms are the most evolutionarily conserved and possess a unique regulatory feature, namely, of regulation by the lipid PI(4,5)P_2 itself. Lipid regulation takes place via the pleckstrin homology (PH) domain of PLCdelta1, a protein module that is characterized by high-affinity binding to the lipid as well as to the water-soluble Ins(1,4,5)P_3. Recent studies have identified a new isoform of PLCdelta, the delta4 enzyme, and indicated that its PH domain, unlike that of delta1, does not bind to Ins(1,4,5)P_3. To understand the role of the PLCdelta4 PH domain in the localization and function of PLCdelta4 enzyme, we compared the inositol-lipid and inositol phosphate binding properties of the enzyme, and its isolated PH domain, with the similar features of the PLCdelta1 protein. Both PLCdelta enzymes showed binding to lipid micelles containing PI(4,5)P_2, but the binding of PLCdelta4 was significantly less sensitive to inhibition by Ins(1,4,5)P_3 than that of PLCdelta1. The isolated PH domain of PLCdelta4, expressed as a GFP fusion protein, also displayed PI(4,5)P_2-dependent binding to lipid micelles as well as high-affinity binding to the soluble Ins(1,4,5)P_3. A 10-fold higher concentration of $InsP_3$ was required to displace the PLCdelta4 PH domain from the lipid vesicles compared with PLCdelta1-PH, with a lower Ins(1,4,5)P_3 affinity reflected in the Ins(1,4,5)P_3 binding assays. Importantly, the concentrations of $InsP_3$ required to displace the full-length proteins from the lipid micelles were significantly higher than the isolated PH-domains with both isoforms of PLCdelta. When expressed in various cells, both PLCdelta1-PH-GFP and PLCdelta4-PH-GFP localized to the plasma membrane, but significantly higher concentrations of the latter were found in the cytosol. PLCdelta4-PH also showed prominent nuclear localization, with bright nucleolar dots that were never detected with the PLCdelta1-PH domain. However, the full-length PLCdelta4 was found to be associated primarily with the endoplasmic reticulum and showed neither a nuclear nor a prominent plasma membrane localization. The data suggest that the PH domain of PLCdelta4 is not responsible for the localization of the enzyme, probably because it is not fully exposed in the molecule. The differential effects of Ins(1,4,5)P_3 on the membrane association and localization of PLCdelta1-PH and PLCdelta4-PH are partly attributable to differences in their PI(4,5)P_2 and $InsP_3$ affinities. Our data also suggest that intramolecular interactions between the PH domain and the rest of the molecule contribute to the different Ins(1,4,5)P_3 sensitivities as well as to the differential localization of these proteins.

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Monitoring phosphatidylinositol 4-kinase activity in single cells using the PH domains of the oxysterol binding protein and the adaptor protein FAPP1

A. Balla, Tujmetova, Tsiomenko, Várnai, T. Balla

Phosphatidylinositol 4-kinases catalyze the first reaction step in the synthesis of phosphoinositides. Four mammalian PI 4-kinases have been identified to date; the type-III enzymes (alpha and beta forms) have similarities to PI 3-kinases within their catalytic domains while the type-II forms (alpha and beta) belong to a new family of lipid kinases. We have previously described the differential cellular localization of these proteins and have been seeking methods by which their reaction product PI(4)P could be detected in single cells. Protein domains, including PH domains, fused to the green fluorescent protein (GFP) have been successfully used to follow localized inositol lipid changes in living cells, an area in which our group has been particularly active. Based on *in vitro* binding assays, we have shown that the PH domains of the oxysterol binding protein (OSBP) and the adaptor protein FAPP1 bind to PI(4)P very specifically. Therefore, we investigated the usefulness of the PH domains of the two proteins to detect PI(4)P in living cells.

When expressed in COS-7 cells, OSBP-PH was associated with the Golgi and with small vesicles scattered around the cytoplasm, a small amount also being found in the plasma membrane. FAPP1-PH was more confined to the Golgi, especially in live cells. Expression of both domains at high levels caused fragmentation and tubulation of the Golgi that was reminiscent of the changes caused by brefeldin A. Although both PH domains showed co-localization with PI4K type-IIIbeta over the Golgi, inhibition of the enzyme by wortmannin did not change the distribution of either PH domains. In cells overexpressing the type-II PI 4-kinases, we observed no co-localization of either PH domains with the enzymes in the endocytic compartments and found only the Golgi-localized fraction of the type-IIbeta enzyme in co-localization with either of the PH domains. Activation of PLC by Ca^{2+} ionophores caused dissociation of OSBP-PH from all membrane sites, a condition that rapidly reversed after restoration of Ca^{2+} levels. The plasma membrane association of OSBP-PH was significantly higher after the Ca^{2+} transient and completely prevented by wortmannin. The data suggest that the localization of the OSBP and FAPP1 PH domains is only partially dependent on PI(4)P and that the Golgi localization of these constructs involves additional, probably protein-protein, interactions. However, the use of these molecular tools has allowed the identification of the type-III PI 4-kinases as the source of PI(4)P formation in the plasma membrane after Ca^{2+}-induced PLC activation.

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PEPTIDE HORMONE RECEPTORS AND SIGNAL TRANSDUCTION

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Elucidation of the mechanisms by which peptide hormones control the cellular responses and signaling pathways of their endocrine and other target cells is the major focus of our research, particularly the characterization of the structure-function properties of specific G protein–coupled receptors (GPCRs) and their cellular processing and signal transduction pathways. We investigate the regulation and structure-function properties of the GPCRs for the central decapeptide reproductive hormone gonadotropin-releasing hormone (GnRH) and the vasoactive octapeptide angiotensin II (Ang II), and address the biochemical mechanisms that mediate their cellular actions. We perform our studies in normal and immortalized hypothalamic neurons, pituitary gonadotrophs, adrenal glomerulosa, and hepatic cells to elucidate the signaling pathways of the agonist-activated GnRH and Ang II receptors and the manner in which they regulate the metabolic, secretory, and growth responses of their respective target cells.

Receptor-mediated regulation of the GnRH pulse generator

Krsmanovic, Navarro, Martinez-Fuentes,[b] *Catt; in collaboration with Mores*

The pulsatile secretory activity of the network of GnRH-producing neurons in the hypothalamus and, consequently, of the GnRH-regulated pituitary gonadotroph cells is essential for maintenance of the gonadotropin secretory profiles that ensure normal reproductive function. An analysis of the cellular and biochemical mechanisms of episodic neurosecretion in fetal hypothalamic cells and GT1-7 neuronal cells revealed that pulsatile GnRH secretion is calcium-dependent and regulated by cyclic AMP and cell excitability. It is also dependent on autocrine agonist activation of the endogenous GnRH receptor (GnRH-R) that we have identified in GnRH neurons and is abolished by GnRH antagonists. The agonist-activated GnRH-R couples primarily to G_q and to G_s and G_i proteins according to the prevailing GnRH concentration. These and other findings indicate that an agonist-induced concentration-dependent switch in coupling of the GnRH-R between specific G proteins regulates $G_{q/11}$-$InsP_3$/Ca^{2+} signaling as well as G_s/cAMP-induced stimulatory and G_i/cAMP-dependent inhibitory responses. Our *in vitro* studies suggest that this autocrine inhibitory mechanism can function as a timer to regulate the frequency of Ca^{2+}- and cAMP-dependent episodes of GnRH release.

In related studies, we have found that hypothalamic GnRH neurons and their immortalized counterparts (GT1-7 cells) express not only nuclear but also cell membrane receptors for the two estrogen receptor subtypes (ERalpha and ERbeta). The membrane-associated ERs expressed in

GnRH neurons undergo high-affinity interactions with adenylyl cyclase–inhibitory G proteins, modulate intracellular cAMP signaling, and regulate the GnRH secretory profile. The sensitivity of this interaction to picomolar estradiol concentrations suggests that the process represents a physiological negative feedback action of estrogen on the GnRH neuron.

Krsmanovic LZ, Mores N, Navarro CE, Arora KK, Catt KJ. An agonist-induced switch in G protein coupling of the gonadotropin-releasing hormone receptor regulates pulsatile neuropeptide secretion. *Proc Natl Acad Sci USA* 2003;100:2969-2974.

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Agonist-induced activation of MAP kinase in GnRH neuronal cells

Shah, Farshori, Catt

GPCRs mediate cellular responses to diverse extracellular messenger molecules. Recent studies have shown that GPCR-mediated signaling pathways include transactivation of the receptor tyrosine kinases (RTKs), epidermal growth factor receptor (EGFR), and receptors for platelet-derived growth factor, neurotrophins, and fibroblast growth factor. The complex cross-communication between GPCRs and RTKs affects sets of signaling molecules primarily determined by cell context and the types of receptors activated, which in turn elicits a variety of cellular effects during development, proliferation, differentiation, survival, repair, and synaptic transmission in the central nervous system.

GnRH uses multiple signaling pathways to activate extracellularly regulated MAP kinases, such as ERK1/2, in normal and immortalized pituitary gonadotrophs and GnRH neurons (GT1-7 cells) and in transfected cells expressing the GnRH receptor. In GT1-7 cells, which express receptors for GnRH and epidermal growth factor (EGF), these agonists and phorbol myristate acetate (PMA) stimulate ERK1/2 phosphorylation. The actions of GnRH and PMA, but not that of EGF, depended on activation of protein kinase C (PKC) and on transactivation and phosphorylation of the EGF receptor. Both GnRH and EGF increased tyrosine-phosphorylation of the EGF receptor. GnRH and PMA, but not EGF, caused rapid phosphorylation of the proline-rich tyrosine kinase Pyk2 at Tyr 402, reactions that depended on Ca^{2+} and activation of PKC. GnRH stimulation caused translocation of PKC alpha and epsilon to the cell membrane and enhanced the association of Src with Pyk2, EGFR, and the activated PKC isoforms. Inhibition of Src kinase and dominant negative Pyk2 attenuated ERK1/2 activation by GnRH and PMA, but not by EGF. These findings indicate that Src and Pyk2 act upstream of the EGFR to mediate its transactivation, which is essential for GnRH-induced ERK1/2 phosphorylation in hypothalamic GnRH neurons.

Related studies analyzed the mechanism of agonist-induced activation of Pyk2 and its relationship with ERK1/2 phosphorylation in non-neuronal HEK293 cells stably expressing the GnRH receptor (GnRHR). In this system, GnRH stimulation caused rapid and sustained phosphorylation of Pyk2 and ERK1/2 accompanied by their nuclear translocation. As expected, Pyk2 was also localized on cell membranes and focal adhesions. However, dominant regulatory Pyk2 had no effect on GnRH-induced ERK1/2 phosphorylation and *c-fos* expression. The actions of GnRH on ERK1/2 and Pyk2 were mimicked by activation of PKC and abolished by its inhibition. GnRH caused translocation of PKC alpha and delta isoforms to the cell membrane as well as phosphorylation of Raf at Ser 338, a major site in the activation of MEK/ERK1/2. Stimulation of HEK293 cells by EGF caused marked ERK1/2 phosphorylation that was mediated by activation of the EGFR receptor, whereas GnRH-induced ERK activation was completely independent of EGFR activation. The results indicate that activation of PKC is responsible for GnRH-induced phosphorylation of both ERK1/2 and Pyk2 in HEK cells and that Pyk2 activation does not contribute to GnRH signaling. Moreover, GnRH-induced phosphorylation of ERK1/2 and expression of *c-fos* in HEK cells is independent of Src and EGFR transactivation and mediated through the PKC/Raf/MEK cascade. The agonist-induced translocation of Pyk2 to the nucleus suggests that it may exert actions therein as well as at the periphery of the cell.

The duration as well as magnitude of MAP kinase activation has been proposed to regulate gene expression and other specific intracellular responses in individual cell types. GnRH-induced activation of ERK1/2 by GnRH is transient in immortalized GT1-7 neurons but sustained in alphaT3-1 pituitary gonadotropes and GnRHR-transfected HEK293 cells. These cell types also expresses the EGFR and respond to EGF stimulation with marked and transient ERK1/2 phosphorylation. However, GnRH-induced ERK1/2 phosphorylation due to EGFR transactivation was confined to GT1-7 cells, in which neither EGF nor GnRHR activation caused nuclear translocation of phospho-ERK1/2 into the nucleus. In contrast, agonist stimulation of GnRHRs expressed in HEK293 cells caused sustained phosphorylation and nuclear translocation of ERK1/2 by a PKC-dependent but EGFR-independent pathway. These and other findings indicate that the duration of ERK1/2 activation depends on the signaling pathways used by GnRH in specific target cells and that transactivation of the tightly regulated EGFR can account for the transient ERK1/2 responses that are elicited by stimulation of certain GPCRs.

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Angiotensin AT$_1$ receptor signaling

Shah, Catt; in collaboration with Gaborik, Hunyady, Olivares Reyes

The type 1 angiotensin II receptor (AT$_1$R) mediates the known physiological actions of the octapeptide hormone angiotensin II (Ang II) on blood pressure regulation, aldosterone secretion, and sodium balance. Recently, in addition to its known involvement in arterial hypertension, Ang II activation of AT$_1$ receptors in the cardiovascular system has been implicated in the development of atheroma and cardiac failure. Most of the actions of Ang II are mediated by coupling of the AT$_1$R to G$_{q/11}$ proteins, followed rapidly by the initiation of phosphoinositide-calcium signaling and activation of PKC isoforms. These actions are associated with the onset of phosphorylation cascades to the nucleus and increased expression of genes regulating cell growth, differentiation, and function. Many of the growth-related actions of Ang II are mediated by transactivation of the EGF receptor (EGFR), which initiates ras-dependent stimulation of MAP kinase cascades. We have analyzed this process in C9 hepatic cells and AT$_1$R-transfected COS-7 cells, in which Ang II-induced ERK phosphorylation proved to be largely dependent on transactivation of the EGFR and independent of the endocytosis of the AT$_1$R and EGFR. In C9 cells, Ang II-induced ERK activation is initiated by a PKCdelta-dependent but Ca^{2+}-independent mechanism and is predominantly mediated by the Src/Pyk2 complex through transactivation of the EGFR. Further investigations are addressing the nature of the interactions between GPCRs and receptor tyrosine kinases and the extent to which caveolae and other cell membrane structures are involved in the signaling cross-talk between different types of plasma-membrane receptors.

In a mutational analysis of the function of a highly conserved domain (DRYXXV/IXXPL) in the second intracellular loop of the AT$_1$R, individual Ala replacements of the Asp^{125} and Arg^{126}, but not of Tyr^{127}, moderately impaired agonist-induced inositol phosphate signaling. However, concomitant Asp/Arg substitutions markedly reduced both inositol phosphate signaling and AT$_1$R internalization. Alanine-scanning showed that Ile^{130}, His^{132}, and Pro^{133} substitutions reduced agonist-induced inositol phosphate signal generation, whereas Met^{134} mutations also impaired receptor internalization. However, the D125A mutant AT$_1$R exhibited moderate constitutive activity, as indicated by increased basal ERK activation and enhanced inositol phosphate responses to partial agonists. Agonist-induced stimulation of the Elk1 promoter showed parallel impairment with inositol phosphate signal generation after mutations in this region of the AT$_1$R. The data suggest that Ca^{2+} signaling is required for the nuclear effects of angiotensin II–induced ERK activation, and are consistent with the role of the conserved DRY sequence in AT$_1$R activation, and of Asp^{125} in constraining the receptor in its inactive conformation. It is likely that an apolar surface that includes Ile^{130} and Met^{134} in the cytoplasmic extension of the third transmembrane helix has a direct role in G protein coupling and signal generation.

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Angiotensin AT$_1$ receptor endocytosis and cellular processing

Shah, Baukal, Catt; in collaboration with Balla, Gaborik, Hunyady, Mihalik[a]

We previously demonstrated that agonist-induced endocytosis of AT$_1$ receptors is a clathrin- and dynamin-dependent process at physiological Ang II concentrations but is independent of these proteins at high levels of agonist stimulation and that Gq-mediated receptor signaling at the membrane level, and to MAP kinase cascades, is independent of receptor endocytosis. Once internalized, Ang II-receptor complexes are processed via endosomes to structures that resemble multivesicular bodies and then return to the cell surface by a rapid recycling pathway that is dependent on PI 3-kinase. The receptor is also recycled via a slower pathway that is less sensitive to inhibition of PI 3-kinase. Related studies also found AT$_1$ receptor endocytosis to be dependent on the interaction of the proline-rich domain of dynamin-2 with SH3 domains of amphiphysins and endophilins, similar to the recruitment of dynamin-1 during the recycling of synaptic vesicles.

In earlier studies, we observed that agonist-induced internalization of the AT$_1$ receptor requires an STL triplet in its cytoplasmic C-terminal region and is associated with phosphorylation of a specific serine-/threonine-rich sequence in this domain. In yeast, hyperphosphorylation of the alpha-factor pheromone receptor has been reported to regulate endocytosis of the receptor by facilitating its monoubiquitylation at lysine residues in its cytoplasmic tail. In our efforts to determine whether this process could contribute to the internalization of the AT$_1$R, we relied on CHO cells to express mutant receptors with deletion or replacement of lysine residues in their agonist-sensitive serine/threonine-rich region. The modifications had no effect on the Ang II-induced receptor endocytosis, and fusion of ubiquitin in-frame to an internalization-deficient truncated AT$_1$ receptor mutant did not restore the endocytosis of the resulting chimeric receptor. After substitution of all lysine residues in the serine/threonine-rich region, we observed no impairment of receptor internalization, when it was performed at saturating angiotensin II concentrations at which endocytosis occurs by a beta-arrestin- and dynamin-independent mechanism. These findings indicate that ubiquitylation of the AT$_1$ receptor is not required for its agonist-induced internalization and suggest that endocytosis of mammalian GPCRs occurs by different mechanisms than those of yeast GPCRs. However, it is possible that ubiquitylation has other functions in the biosynthesis and trafficking of the AT$_1$R and other GPCRs

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STRUCTURE AND FUNCTION OF PEPTIDE AND PROTEIN HORMONES

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Our research program is concerned with the analysis, synthesis, protein expression, and structure-function relationships of peptide and protein hormones. We are currently focusing on the structure and function of the angiotensin II (Ang II) and gonadotropin-releasing hormone (GnRH) receptors and related studies. Our major accomplishments over the past year include the development of mass spectrometric analysis for the identification of serine and threonine phosphorylation sites, verification of corticotropin-releasing hormone (CRH) production in an immortalized rat hypothalamic cell line, and determination of a glycosylation site of pigeon IgG.

Mass spectrometric analysis of phosphopeptides
Li, Wang, H-C. Chen
Protein phosphorylation is considered the most important reaction in the regulation of eukaryotic cell function. Of the known proteins phosphorylated, 99 percent are phosphorylated at serine or threonine. We have developed a chemical method that converts phospho-Ser and phospho-Thr to cysteic acid and beta-methyl cysteic acid, respectively, for direct sequencing of the Ser and Thr phosphorylation sites by electrospray ionization (ESI) tandem mass spectrometry. The conversion can be carried out at 1 microM concentration of the peptide. We showed that the cysteic acid and beta-methyl cysteic acid residues in the sequence are both stable and easily identifiable in a series of *y* and *b* ions under general conditions for tandem mass spectrometric sequencing applicable to common peptides. However, the reactivity of the free hydroxyl group on serine and threonine by sodium hydroxide–induced beta-elimination has not been critically examined. Under the widely used conditions previously reported by others for beta-elimination and ethanethiol addition, two analogous phosphopeptides (KMpSTLSYR and KMSpTLSYR) yielded an ethylthio group with a mass 44 in excess of that expected for the modified phosphopeptide analyzed by MALDI-TOF. ESI tandem mass spectrometric analysis confirms that the modification occurred on the hydroxyl group of Ser and Thr in addition to P-Ser and P-Thr. On the other hand, we could not detect modification on the free hydroxyl group of Ser or Thr when conditions were mild, *viz*, 0.1 N NaOH/0.6 M sodium sulfite at 25°C for 24 hours. This finding suggests that temperatures above 25ºC and excessive alkalinity should be avoided in order to prevent the beta-elimination of the hydroxyl group of Ser and Thr in peptides. The outcome is of particular concern when employing highly sensitive tandem mass spectrometric methods for the identification and localization of Ser and Thr as modification sites by the beta-elimination/Michael addition reaction. The additional modification site(s) may complicate the interpretation of data and lead to an erroneous conclusion.

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Verification of CRH production in an immortalized rat hypothalamic cell line

Morell, H-C. Chen

Previously, we chemically synthesized a 41-residue peptide with a sequence corresponding to human/rat CRH. Brief oxidation of the CRH peptide in dilute hydrogen peroxide and acetic acid solution yielded Met(O)-CRH at positions 21 and 38. We used the two peptides to develop a reverse-phase HPLC system that permitted base-line separation between the two. To verify the production of CRH in a clonal cell line (designated as IVB) derived from rat embryonic hypothalamic tissue, we fractionated cell lysates by the HPLC system and analyzed the aliquots by a specific radioimmunoassay for rat CRH. The endogeneous immunoreactivity coeluted with synthetic CRH. Furthermore, oxidation of the lysate converted its radioimmunoreactivity elution position to one coincident with that of the oxidized CRH separated from CRH. In addition, IVB cells exhibited high-affinity binding of CRH and shared many functional characteristics with other hypothalamic CRH cells. This new immortalized cell line may be a useful model for understanding the molecular mechanisms that operate in hypothalamic CRH neurons.

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Identification of pigeon IgG and its N-glycopeptide

H-D. Chen, H-C. Chen

Among glycoproteins, those from the pigeon egg were found uniquely to possess binding activity for uropathogenic *Eschericia coli* and *Shigella suis*. Previously, we purified and sequenced four components from pigeon egg white proteins and identified them as ovomucoid, ovotransferrin, and two ovalbumins. All four proteins contain a terminal Gal-alpha 1,4-Gal sequence that is uncommon in mammals and in other avians. We purified a major pigeon serum glycoprotein, IgG (alias IgY). N-terminal sequence analyses of the light chain and heavy chain confirmed it as IgG based on its homology to the corresponding regions of chicken, duck, and human IgG. Furthermore, the heavy chain of pigeon IgG, but not the light chain, stained with GS-1 lectin and anti-P1 mAb (specific for P1 blood type), indicative of a distinctive Gal-alpha 1,4-Gal linkage on the termini of the glycan that facilitates binding to uropathogenic microbes. Using reverse-phase HPLC, we isolated one glycopeptide from a tryptic digest of the reduced-pyridinylethylated heavy chain. Peptide sequencing of this glycopeptide before and after glycoamidase F treatment revealed an N-glycosylation site in the sequence that is homologous to that located at the CH3 domain of chicken and duck IgG. Given that both avian and mammalian IgG are known to contain two N-glycosylation sites, a search of the second glycopeptide of pigeon IgG is under way.

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GONADAL RECEPTORS AND MECHANISM OF ACTION OF PEPTIDE HORMONES IN STEROIDOGENIC CELLS

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We investigate the molecular basis of peptide hormone control of gonadal function, with particular emphasis on the structure and regulation of luteinizing hormone and prolactin receptor genes and on the regulatory mechanisms involved in the hormonal control of steroidogenesis. Our studies focus on the regulation of the promoter domain of the LH receptor gene and its control by both nuclear orphan receptors (histone acetylation, deacetylation, and methylation) and second messengers as well as on the complex genomic structure of the prolactin receptors (PRLR) and the multiple control of their transcription. We are particularly interested in the function of two novel short forms of alternative spliced transcripts of the prolactin receptors and their relevance to physiological regulation and breast cancer. We also investigate hormone-regulated membrane coupling and intracellular events involved in the modulation of steroid biosynthesis in the testis as well as novel gonadotropin-regulated genes of relevance to testicular and ovarian function and other reproductive processes.

Transcriptional regulation of the LH receptor

Zhang, Fatima, Dufau

The luteinizing hormone receptor (LHR), a G protein–coupled receptor essential for reproductive function and predominantly located in the plasma membrane of gonadal cells, mediates gonadotropin signaling and triggers intracellular responses that participate in gonadal maturation and function and the regulation of steroidogenesis and gametogenesis. We previously demonstrated regulation of the Sp1/Sp3-driven TATAless 180 bp promoter of the human LH receptor (hLRH) by the nuclear orphan receptors EAR2 and EAR3/COUP-TF1 (inhibitory) and TR4 (stimulatory), which bind competitively and with high affinity to an imperfect direct-repeat motif composed of an estrogen response element half-site and a second degenerate half-site (DR) located within the promoter 5′ of two functional SP1/SP3 sites and exhibit differential binding to the rat and human LHR promoters. EAR2 and EAR3/COUP-TFI bind with two- to three-fold higher affinity to the human promoter than to the rat promoter because of the presence of a G 3′ to the second DR site in the human sequence, which causes weaker inhibition of the rat promoter activity by these orphan receptors. In addition, TR4 is not stimulatory in the rat owing to a single base-pair mismatch in the second DR site that abolishes its binding. Changes in endogenous levels of EAR2 and EAR3 during gonadotropin stimulation of rat granulosa cells in culture correlate with derepression of promoter activity.

We recently reported that local chromatin structure modulated by histone acetylation and deacetylation plays a role in human LHR gene regulation, namely, the most proximal Sp1(I) site of the two active Sp1/Sp3 sites of the hLHR gene promoter serves as a docking site to recruit a histone deacetylase–mSin3A complex to the promoter, which causes silencing of the LHR gene via histone hypoacetylation–induced chromatin condensation, a mechanism that is independent of the pathway involving unliganded hormone receptors/orphan receptors and co-repressor NcoR/SMRT. However, this and/or epigenetic chromatin modifications may be involved in the second messenger activation of hLHR expression, which we are currently investigating.

We aim to establish the functional connection between orphan receptors and the Sp1/Sp3 complex and their impact on the basal transcription machinery. Given that the orphans operate in repressive/inductive states of the hLRH of the granulosa cells of early follicles (repressive) but not in those of mid- to late-ovulatory follicles and luteal follicles (relieved of repression by reduction of the expressed orphan inhibitors), the studies are relevant to understanding hLHR orphan control during the menstrual cycle at different stages of granulosa/luteal cell development.

We identified the proximal Sp1(I) site as critical for the EAR2, EAR3/COUP-TFI repression in both human and rat, as its mutation reduced inhibition by EAR2 and abolished inhibition by EAR3/COUP-TFI (EAR3). With EAR3 exerting a complete Sp1/Sp3-dependent inhibitory effect, cotransfection analyses in SL2 cells showed that both Sp1 and Sp3 are required for repression. Mutual recruitment of EAR3 and Sp1/Sp3 bound to their cognate sites further supported functional cooperation between Sp1 and DR domains. Deletion of the EAR3 N-terminal and DNA binding domains that reduced EAR3's interaction with Sp1 also reduced its inhibition on hLHR gene transcription. Furthermore, we demonstrated interaction of TFIIB with Sp1/Sp3 at the Sp1(I) site, in addition to its association with EAR3/COUP-TFI and the TATA-less core promoter region. The interaction relied on Sp1 site–bound Sp1/Sp3 complex and adaptor protein(s) present in JAR cell nuclear extracts. We further demonstrated that EAR3 specifically reduced association of TFIIB with the Sp1(I) site without interfering in EAR3's interaction with the hLHR core promoter. The C-terminal region of EAR3, which did not participate in its interaction with Sp1, was required for its inhibitory function and may affect the association of TFIIB with Sp1. Moreover, perturbation of the association of TFIIB with Sp1 by EAR3 was reflected in reduced recruitment of RNA PoL II to the promoter. Overexpression of TFIIB counteracted the inhibitory effect of EAR3 and activated hLHR gene transcription in an Sp1-site dependent manner. These findings indicate that TFIIB is a critical component in regulatory control of EAR3 and Sp1/Sp3 on the initiation complex. Such

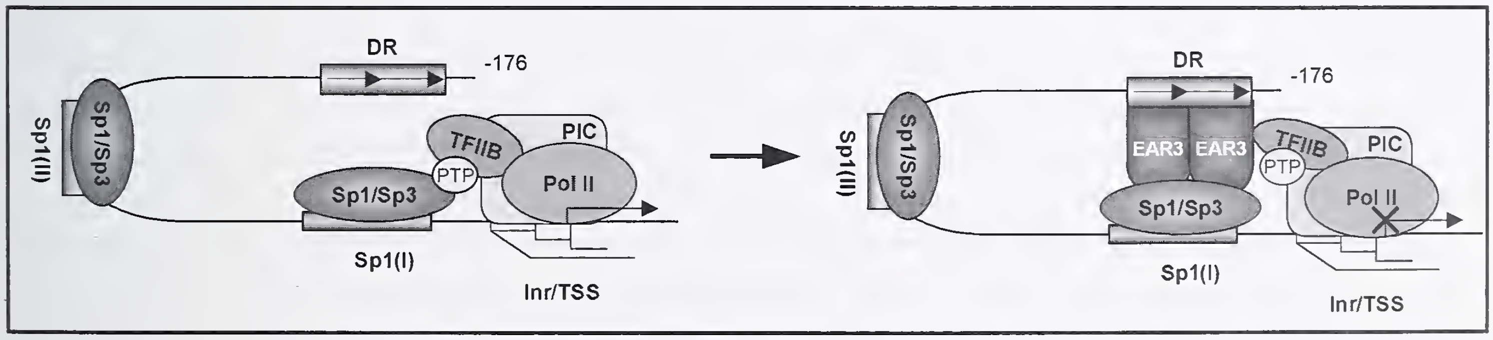

FIGURE 4.1

Mechanism of Sp1 (I) site–dependent silencing of hLHR gene transcription by EAR3/COUP-TFI

EAR3/COUP-TFI bound to the DR motif interacts with Sp1/Sp3 bound to the Sp1(I) site. This interaction significantly impairs the robust association of TFIIB to the Sp1(I) site without affecting the recruitment of TFIIB to the hLHRgene core promoter region. Anchoring of TFIIB at the Sp1(I)site does not require earlier binding of EAR3 to its cognate site, as the association is present in the absence of EAR3. Interaction of TFIIB with Sp1/Sp3 is indirectly bridged by a currently unidentified protein(s) (PTP). The EAR3-reduced association of TFIIB to the Sp1/Sp3-DNA complex induces a non- or less productive form of preinitiation complex, in which the recruitment of RNA POLII to the hLHR promoter decreased when the hLHR gene was subjected to a repressed state by EAR3 in JAR cells.

PIC: preinitiation complex; Inr: initiator element; TSS: transcriptional start site; PTP: putative tethering protein. Sp1-1 and Sp1-2 are functional sites for promoter activity. Both sites bind Sp1 and Sp3 and contribute to basal promoter activity. DR: imperfect estrogen receptor half-site direct repeat site for EAR2, EAR3/COUP-TF1 binding/function (inhibitory), and TR4 (stimulatory). TSS: transcriptional start sites vertical lines.

cross-talk among EAR3, TFIIB, and Sp1/Sp3 reveals that repression of hLHR gene transcription by nuclear orphan receptors is achieved via perturbation of communication between Sp1/Sp3 at the Sp1-1 site and the basal transcription initiator complex.

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Cell-specific and hormone-regulated expression of gonadotropin-regulated testicular RNA helicase resulting from alternative utilization of translation initiation codons in the rat testis

Sheng, Tsai-Morris, Lee, Dufau

The gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) cloned from rat Leydig cell, mouse testis, and human testis cDNA libraries is a novel member of the DEAD-box protein family of RNA helicases that possess ATPase and RNA helicase activity and is the first member found to be regulated by a hormone. The mRNA of the GRTH gene is transcriptionally up-regulated by gonadotropin (hCG) via cyclic AMP–induced androgen formation in testicular Leydig cells. To evaluate protein levels of the GRTH gene regulated by gonadotropin, we raised a specific rabbit polyclonal antibody to a GRTH peptide. Immunocytochemical and Western blot analysis identified the GRTH protein as a developmentally regulated protein in Leydig cells and in germ cells (pachytene spermatocytes and round spermatids) of the rat testis. Recent studies have identified three ATGs with potential for the generation of multiple species. Germ cells primarily used the first ATG codon (+1) and contained major proteins of 61/56 kDa species, whereas Leydig cells preferentially used the second ATG codon (+343) with expression of 48/43 kDa species. A third ATG was weakly used and yielded a 33 kDa species only in germ cells. The androgen receptor antagonist flutamide prevented an increase in GRTH 43-kDa protein in Leydig cells caused by hCG treatment. In round spermatids, hCG caused a significant decrease of 61 kDa species and an induction of 48/43 kDa species, whereas we observed no changes in pachytene spermatocytes. Reversal of this hormone-induced switch of expression by flutamide indicated a role of androgen in utilization of the second ATG. Our studies have demonstrated a cell-specific and hormone-dependent alternative usage of ATG codons in the testis. They have also revealed that both the androgen-dependent alternative use of ATG codons in the rat testis and the androgen-dependent transcription of GRTH expression are accompanied by a marked increase in the 43-kDa species. The Leydig cell is tonically regulated by gonadotropin, which promotes testosterone formation in these cells. Autocrine actions of testosterone in Leydig cells and paracrine actions at tubule sites (round spermatids) cause increases in GRTH and promote the utilization of the second ATG codons in both cells types. These findings indicate that the expression of GRTH proteins is regulated by gonadotropin/androgen at the translational level.

Sheng Y, Tsai-Morris C-H, Dufau ML. Cell-specific and hormone-regulated expression of gonadotropin-regulated testicular RNA helicase gene (GRTH/Ddx25) resulting from alternative utilization of translation initiation codons in the rat testis. *J BiolChem* 2003;278:27796-27803.

Prolactin receptors

Qazi, Meng, Dong, Tsai-Morris, Dufau

Prolactin is a polypeptide hormone with highly diversified biological actions in reproduction, lactation, maternal behavior, steroidogenesis, growth, metabolism, water-salt balance, and immune regulation. Its actions are mediated by a single-transmembrane domain receptor of the cytokine/ growth hormone family. The expression of the human prolactin receptor is controlled by multiple promoters that use distinct mechanisms to regulate gene expression. The human gene uses five promoters for transcription of noncoding exons-1 as well as a generic promoter/exon-1 termed hPIII/E13, which is present in rodent and human and appears to be the most commonly used promoter. Prolactin stimulates the proliferation of human breast cancer cells and exhibits additive effects to that of estrogen in T-47D and MCF7 cells. Estradiol activates the hPIII promoter in a dose-dependent manner, an action that is prevented by the specific estrogen receptor antagonist ICI 182,780. Further, estradiol stimulates the expression in T-47D cells of two prolactin receptor isoforms, the generic form and a human-specific form with alternative exons-1. These findings support the role of estradiol in regulating human prolactin receptor expression in human breast cancer cells. The receptor is widely distributed and exists in several forms, including long and intermediate forms as well as two short forms identified in our laboratory as S1a and S1b, which are derived from alternative splicing and possess a truncated intracellular domain. The truncated forms are dominant-negative repressors of the function of the long form and are present in several normal tissues and human breast cancer and cell lines. We have demonstrated differential expression of these forms in cancer profiling arrays and by real-time PCR quantitation using human breast tissues. Both approaches revealed significant decreases in the ratio of short to long forms in the breast tumor tissue. Given that the short forms act as dominant negative regulators of the stimulatory actions of the long form *in vitro,* their relatively reduced expression in cancer could cause unopposed prolactin-mediated stimulatory actions of the long form.

Acting through its cognate receptor, prolactin activates the tyrosine kinase Jak2, which is essential for prolactin-induced differentiation and the activation of Stat5 in mammary epithelial cells. Receptor dimerization can be either constitutive or ligand-induced. Recognizing that the action of the short forms could result from their heterodimerization with the long form of the receptor, we studied the complex formation between the short and long isoforms. To investigate the formation of homodimers and heterodimeric forms of the long and short forms of the receptor, we used 3′ tagged fusion constructs of various prolactin receptor forms with V5, FLAG, or GFP. As a 3′ tagged construct transfected into HEK293 cells, the long form exhibited prolactin-induced beta-casein activity comparable to the wild type. In addition, when each of the 3′ tagged short forms of receptor was cotransfected with the long forms, the wild-type or the 3′ tagged constructs inhibited prolactin-induced activation of the beta-casein promoter activity by the long form of the receptor. The long form formed a homodimer under nonreducing conditions while the monomer was detected under reducing conditions, with prolactin not affecting the homodimerization. Similarly, under nonreducing conditions, S1a or S1b isoforms formed a homodimer unaffected by the presence of prolactin. Coimmunoprecipitation of the cotransfected long form and each of the 3′ tagged short forms (S1a and S1 b) in HEK-293 cells showed heterodimerization of the individual short forms with the long form. We thus conclude that human prolactin receptors can form homodimers (long form, S1a and S1b) independent of ligand treatment. We also demonstrated that heterodimerization between the long and short forms of the receptor is ligand-independent and propose that prolactin receptor homo- and/or heterodimers are constitutively present. Furthermore, we conclude that the association between two different short isoforms of the prolactin receptor could exert a modulatory role on prolactin-induced signaling through the long form of the receptor. The hormonal stimulus could act on the preformed homodimer of the long form to induce the signal by causing changes in conformation of the cytoplasmic domain. Heterodimerization with either short form could abrogate the structural changes induced by the hormone that are required for activation of the dimeric long form of the receptor.

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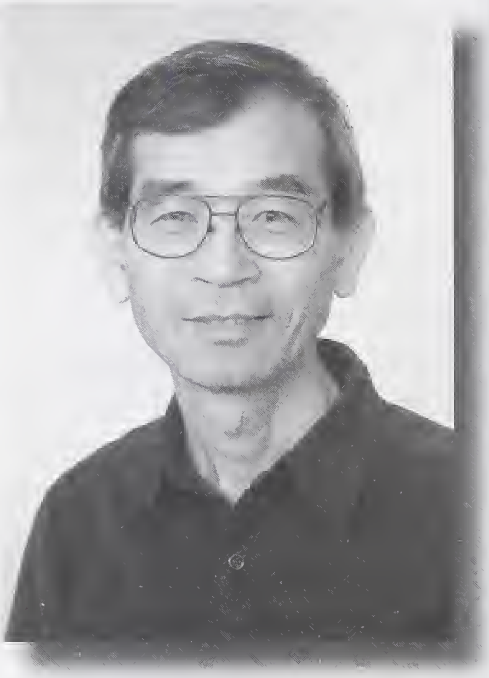
SIGNAL TRANSDUCTION IN SYNAPTIC TRANSMISSION AND PLASTICITY



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We investigate signal transduction mechanisms involved in the enhancement of synaptic transmission and plasticity. Studies of these neural processes are essential to understanding the complexities of learning and memory. Our approach is to generate genetically modified mice by deletion or expression of a gene specifically expressed in the brain. These efforts have resulted in a strain of mice lacking a neural-specific protein, neurogranin (Ng), which is normally expressed at high levels in selective neurons within cerebral cortex, hippocampus, and amygdala and has been implicated in the modulation of synaptic plasticity. Ng binds to calmodulin (CaM) in an inverse Ca^{2+}-sensitive manner; namely, its binding affinity for CaM decreases with increasing Ca^{2+} concentration. It is believed that, at basal physiological levels of Ca^{2+}, all CaM is sequestered by Ng and, upon synaptic stimulation, the influxed Ca^{2+} will displace Ng from the Ng/CaM complex to form Ca^{2+}/CaM and free Ng. The buffering of CaM by Ng serves as a mechanism to regulate neuronal free Ca^{2+} and Ca^{2+}/CaM concentrations. The goals of our project are to define the regulatory function of Ng and to design therapeutic approaches for improving memory in the aging human population and patients suffering from dementia.

Neurogranin enhances hippocampus-dependent learning and memory by promoting calcium-mediated signaling

F. Huang, Wu, K. Huang, Li ; in collaboration with Balschun, Jäger, Reymann*

The neuronal CaM-binding protein Ng, which binds to CaM at the low concentrations of Ca^{2+} normally found under basal physiological conditions, is localized in the neuronal soma and dendrites and found in particularly high concentrations in the dendritic spines. It has been suggested that Ng modulates synaptic responses by buffering and sequestering CaM to regulate the level of free Ca^{2+} and Ca^{2+}/CaM complexes. The hippocampal levels of Ng in adult $Ng^{+/+}$ mice range from 160 to 330 pmol/mg, which is one of the highest levels among neuronal CaM-binding proteins, whereas, in $Ng^{+/-}$ mice, they range between 60 and 240 pmol/mg protein. Induction of long-term potentiation (LTP) caused rapid phosphorylation of the protein by PKC in the hippocampal CA1 region. When tested in the Morris water maze, $Ng^{+/-}$ mice showed significant relationships between the levels of hippocampal Ng and their performance; however, such relationships were less significant among $Ng^{+/+}$ mice whose Ng levels apparently were near the threshold value for optimal performance of such tasks. $Ng^{-/-}$ mice performed poorly in all spatial tasks for which they were tested and exhibited deficits in LTP and concurrent CaMKII autophosphorylation. The frequency response curve of $Ng^{-/-}$ mice was shifted to the right compared with that of the $Ng^{+/+}$ mice; low- frequency stimulations (5 to 10 Hz) induced LTD in the former and modest LTP in the latter. Tetanic stimulation that evoked strong LTP (100 Hz, 1s) displayed a significantly greater Ca^{2+}-transient amplitude in the CA1 pyramidal neurons of $Ng^{+/+}$ than those of $Ng^{-/-}$ mice. However, the $[Ca^{2+}]_i$ decay time constants were similar. The Ca^{2+}-transient amplitudes following weak LTP (100Hz, 400 ms) and long-term depression (LTD) (5 Hz, 3 min) were also greater in $Ng^{+/+}$ than in $Ng^{-/-}$ mice, albeit less significantly so. The diminished Ca^{2+} dynamics in the $Ng^{-/-}$ mice is a likely cause for the animals' decreased propensity to undergo LTP. Thus, the higher the Ng concentration, the greater is the number of Ng/CaM complexes that will form, effectively raising $[Ca^{2+}]_i$ at any given Ca^{2+} influx to promote a more potent signal amplification to enhance synaptic plasticity and learning and memory.

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Participation of NMDA-mediated phosphorylation and oxidation of neurogranin in the regulation of Ca^{2+}- and Ca^{2+}/calmodulin-dependent neuronal signaling in the hippocampus

Wu, K. Huang, F. Huang

Activation of the N-methyl-D-asparate (NMDA) receptor is one of the critical steps leading to the enhancement of synaptic plasticity that underlie learning and memory. Calcium influx induced by the activation of this receptor triggers the stimulation of PKC, Ca^{2+}/CaM-dependent protein kinases, adenylyl cyclases, and nitric oxide synthase. It is the activation of these Ca^{2+}- and Ca^{2+}/CaM-dependent enzymes and their downstream targets that contributes to the NMDA receptor–dependent plasticity. Stimulation of PKC causes phosphorylation of Ng, which promotes the release of Ca^{2+} from intracellular stores through G protein–coupled phosphoinositide second-messenger pathways. In addition, Ng is susceptible to oxidant-mediated modification, which causes thionylation and/or formation of intramolecular disulfide bonds. The latter modification, resembling PKC-mediated phosphorylation, also results in a reduction of binding affinity of CaM. Using mouse hippocampal slices, we demonstrated that NMDA induced rapid and transient phosphorylation and oxidation of Ng. NMDA also caused activation of PKCs, as evidenced by their phosphorylation, whereas such activations were greatly reduced in Ng knock-out mice. A higher degree of phosphorylation of Ca^{2+}/CaM-dependent kinase II and activation of cAMP-dependent protein kinase were also in greater evidence in the wild-type than the knock-out mice. Phosphorylation of downstream targets, including mitogen-activated protein kinases and cAMP response element–binding protein, were significantly attenuated in the knock-out mice. The results suggest that, by its inverse Ca^{2+}-sensitive CaM-binding feature and through its phosphorylation and oxidation, Ng regulates the Ca^{2+}- and Ca^{2+}/CaM-dependent signaling pathways subsequent to the stimulation of NMDA receptor. We hypothesize that the derangement of hippocampal signal transduction cascades in Ng knock-out mice causes the deficit in synaptic plasticity and the defects in cognitive behaviors in these mice.

Wu J, Huang K-P, Huang F. Participation of NMDA-mediated phosphorylation and oxidation of neurogranin in the regulation of Ca^{2+}- and Ca^{2+}/calmodulin-dependent neuronal signaling in the hippocampus. *J Neurochem* 2003;86:1524-1533.

Effect of environmental enrichment on the cognitive behaviors of neurogranin knock-out mice

F. Huang, Wu, Kolker, Park, K. Huang

Environmental enrichment and exercise can alter neural plasticity and improve cognitive performances of rodents as a result of increasing neurogenesis. Neurogranin (Ng) knock-out mice exhibit severe deficits in learning and memory of spatial tasks, induction and maintenance of LTP, and amplification of Ca^{2+}-mediated signaling in hippocampus. To determine if increasing physical activities of these mutant mice can ameliorate their deficits, groups of mutant mice and their wild-type littermates were housed for three weeks in roomier cages, each with several toys and a running wheel for voluntary exploration and exercise. The control groups of both genotypes were housed in the same type of cages without any toy. The wild-type mice in the enriched environment performed significantly better than their controls in both the hidden platform and probe trial versions of the Morris water maze. However, environmental enrichment had no effect on the performance of the mutant mice on these tasks. Hippocampal LTP in the CA1 region induced by high-frequency stimulation was also increased in the enriched group as compared with the control group, whereas no significant change was observed among the mutants. Quantification of the hippocampal levels of Ng by immunoblot revealed that the Ng levels among the wild-type mice housed in the enriched environment were significantly greater than those of the controls. These findings show that environmental enrichment can augment the hippocampal level of Ng, the extent of LTP, and performance in cognitive tasks. Without Ng, as in the case of Ng knockouts, an increase in physical activities has minimal effects on neural plasticity and cognitive behavior.

Role of PKC gamma in the phosphorylation of neurogranin: studies with PKC gamma knock-out mice

K. Huang, Wu, F. Huang

PKC gamma is one of the major Ca^{2+}/phospholipid/diacylglycerol-activated isozymes and is expressed at a high level in the neurons of mammalian brain. Subcellularly, this kinase is localized in the cell bodies, axons, and dendrites but not in the presynaptic terminals. Among all the PKCs, the cellular localization and patterns of developmental expression of PKC gamma resemble those of Ng more closely than those of other PKC isoforms. In the hippocampus, PKC gamma is associated with the NMDA receptor–PSD-95 complexes and is believed to participate in NMDA receptor–mediated signal transduction and enhancement of synaptic plasticity. This brain-specific PKC gamma was believed to be the kinase responsible for the phosphorylation of Ng, an event that has been implicated in signal amplification during synaptic transmission. However, deletion of PKC gamma in mice resulted in only a mild modification of hippocampal LTP induced by high-frequency stimulation and had no effect on synaptic transmission, paired-pulse facilitation, long-term depression, or phorbol-induced LTP. To determine the specificity of the various PKCs in the phosphorylation of Ng and the role of Ng phosphorylation in signal transduction, we investigated the phosphorylation of Ng by PKCs *in vitro* and in hippocampal slices from the wild-type and PKC gamma null mutant mice. *In vitro*, Ng is a substrate of the Ca^{2+}-activated the PKC alpha, beta, and gamma group of isoforms but is poorly phosphorylated by the Ca^{2+}-insensitive delta, epsilon, zeta, and eta group of isoforms; the activities of the latter group of PKCs were less than one-third those of the former. Both the glutamate- and NMDA-stimulated phosphorylations of Ng in hippocampal slices of PKC gamma knock-out mice were lower than those of the wild type. In contrast, the phorbol ester– and high-frequency-tetanus–induced phosphorylations of Ng were comparable between the PKC gamma knock-out and wild-type mice. The findings suggest that activation of PKC gamma is closely coupled to glutamate receptors, whereas phorbol ester and high-frequency tetanic stimulation cause a global activation of multiple PKCs to phosphorylate Ng. The compensatory effects of other PKCs provide a logical explanation for the mild deficits in synaptic plasticity and cognitive behaviors observed in PKC gamma knock-out mice.

Wu J, Li J, Huang K-P, Huang FL. Attenuation of protein kinase C and cAMP-dependent protein kinase signal transduction in the neurogranin knockout mice. *J Biol Chem* 2002;277:19498-19505.

Physiological implication of generating potent thionylating agents, disulfide S-monoxide, and disulfide S-dioxide from oxidation of sulfhydryl compounds by Fenton's reaction

K. Huang, F. Huang

Redox regulation through modification of proteins has emerged as one of the major cellular responses to oxidative and nitrosative stresses. In the CNS, neurotransmission under normal or pathological condition generates a variety of oxidants. Cellular sulfhydryl compounds are the major reductants to protect against oxidative stress, which is detrimental to the survival of cells if not properly suppressed. Numerous neural diseases, including Alzheimer's and Parkinson's diseases, are believed to result in part from excessive damage to neurons by the endogenously produced oxidants in the presence of iron. It has been suggested that the pivotal role of iron in pathogenesis derives from its participation in Fenton's reaction, which generates a highly reactive and toxic hydroxyl radical via reaction of Fe^{2+} and hydrogen peroxide. Oxidation of sulfhydryl compounds by oxidants generated from Fenton's reaction is generally thought to result in disulfides. Detailed analysis of the oxidation products of glutathione and the commonly prescribed sulfhydryl-containing antihypertensive drug captopril by hydrogen peroxide and iron revealed that, in addition to each of their respective disulfides, both the disulfide S-monoxide and disulfide S-dioxide of these two compounds were formed. We purified the oxidized products by reverse-phase HPLC and identified them by mass spectrometry. The disulfide oxides can also be generated by oxidation of sulfhydryl compounds with methyltrioxorhenium (CH_3ReO_3). Both disulfide S-monoxide and S-dioxide are highly reactive toward free sulfhydryl groups to form mixed disulfide; the reaction rate of the latter is much faster than the former when tested by reacting with 5-mercapto-2-nitro benzoate. Both compounds are potent thionylating agents for proteins, which cause reversible modifications of Ng and PKC *in vitro*. Thionylation of Ng attenuates its phosphorylation by PKC, and thionylation of PKC inhibits the kinase activity, suggesting that oxidants may reduce the phosphorylation of Ng through concerted thionylation of both Ng and PKC. Treatment of rat brain slices with oxidants resulted in a rapid thionylation of Ng, which contains four reactive cysteine residues susceptible to modification. The findings indicate that sulfhydryl compounds can protect cells from damage caused by Fenton's reaction through generation of disulfide oxides for reversible modification of proteins. Furthermore, thionylation of proteins serves as a mechanism to transduce the oxidant-mediated signal by modulation of enzymatic activity.

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CALCIUM SIGNALING AND CALCIUM-CONTROLLED CELLULAR FUNCTIONS

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We investigate the cellular signaling cascades in endocrine and neuroendocrine cells and the interactions between plasma membrane electrical events and receptor-controlled pathways. Our main objective is to elucidate the receptors and channels involved in calcium signaling and the role of calcium ions as messengers in control of signaling, secretion, and gene expression. Our approach has been to characterize calcium signaling from a biophysical, physiological, pharmacological, and molecular biology point of view. More recently, we have begun to address how the calcium signaling function in endocrine and neuroendocrine cells is related to the structures of individual receptors and channels. Our current investigations focus on the calcium signaling function of nucleotide ("purinergic") receptors, cyclic nucleotide–gated channels, and several G protein–coupled receptors.

Nucleotide-controlled calcium signaling and secretion
He, Tomic, Gonzales-Iglesias, Yan, Stojilkovic; in collaboration with Koshimizu, Zemkova
Anterior pituitary cells secrete ATP and express nucleotide-gated G protein–coupled P2 receptors (P2YRs) and cation-conducting channels (P2XRs). However, the identification of P2R subtypes and their native ligands and the distribution and function of these receptors within the secretory and nonsecretory pituitary cells remain incompletely characterized. Over the past year, we focused on the lactotroph subpopulation of cells. Our results showed that ATP and ADP, but not UTP and UDP, triggered calcium signaling in a majority of lactotrophs and prolactin (PRL) release in mixed pituitary cells. Consistent with the role of P2Rs in signaling and secretion, the actions of ATP and ADP were abolished in the presence of apyrase, an ectonucleotidase. We identified transcripts for G_q-coupled calcium-mobilizing $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, and $P2Y_6R$, as well as for G_i-coupled $P2Y_{12}R$, in mixed anterior pituitary cells. The ligand selectivity profile of calcium mobilization–dependent signaling and PRL secretion and the blockade of these responses by pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulphonic acid indicated that $P2Y_1R$ mediates the stimulatory action of ATP and ADP. Pituitary cells also express $P2X_{2a}R$, $P2X_{2b}R$, $P2X_3R$, $P2X_4R$, and $P2X_7R$. Several lines of evidence suggested that the $P2X_4R$ subtype provides a major pathway for calcium influx–dependent signaling and PRL secretion. In ongoing experiments, we are focusing on P2R subtypes expressed in gonadotrophs and somatotrophs and on ectoATPase subtypes expressed in pituitary cells.

Recombinant rat P2XR channels differ with respect to their ligand preferences and channel kinetics during activation, desensitization, and recovery. However, the calcium signaling function of P2XRs and the contributions of distinct receptor subdomains to the subtype-specific behavior have been incompletely characterized. We studied the spatiotemporal characteristics of intracellular calcium signaling and its dependence on current signaling in excitable GT1 and nonexcitable HEK293 cells expressing wild-type and chimeric P2XRs. In both cell types, P2XR generated depolarizing currents during the sustained ATP stimulation, which desensitized in the following order (from rapidly desensitizing to nondesensitizing): $P2X_3R > P2X_{2b}{+}X_4R > P2X_{2b}R > P2X_{2a}{+}X_4R > P2X_4R > P2X_{2a}R > P2X_7R$ (see Figure 4.2). HEK293 cells were not suitable for studies on P2XR-mediated calcium influx because of the coactivation of endogenously expressed calcium-mobilizing purinergic P2YRs. However, when expressed in GT1 cells, all wild-type and chimeric P2XRs responded to agonist binding with global calcium signals, which desensitized in the same order as current signals but in a significantly slower manner. The global distribution of calcium signals was present independent of the rate of current desensitization. The temporal characteristics of calcium signals were not affected by voltage-gated calcium influx and removal of extracellular sodium. Calcium signals reflected well the receptor-specific EC_{50}s for ATP and the extracellular zinc and pH sensitivities of P2XRs. The results indicate that, when the host cells do not express other members of nucleotide receptors, intracellular calcium measurements are useful for characterizing the pharmacological properties and messenger functions of P2XRs as well as the kinetics of channel activity.

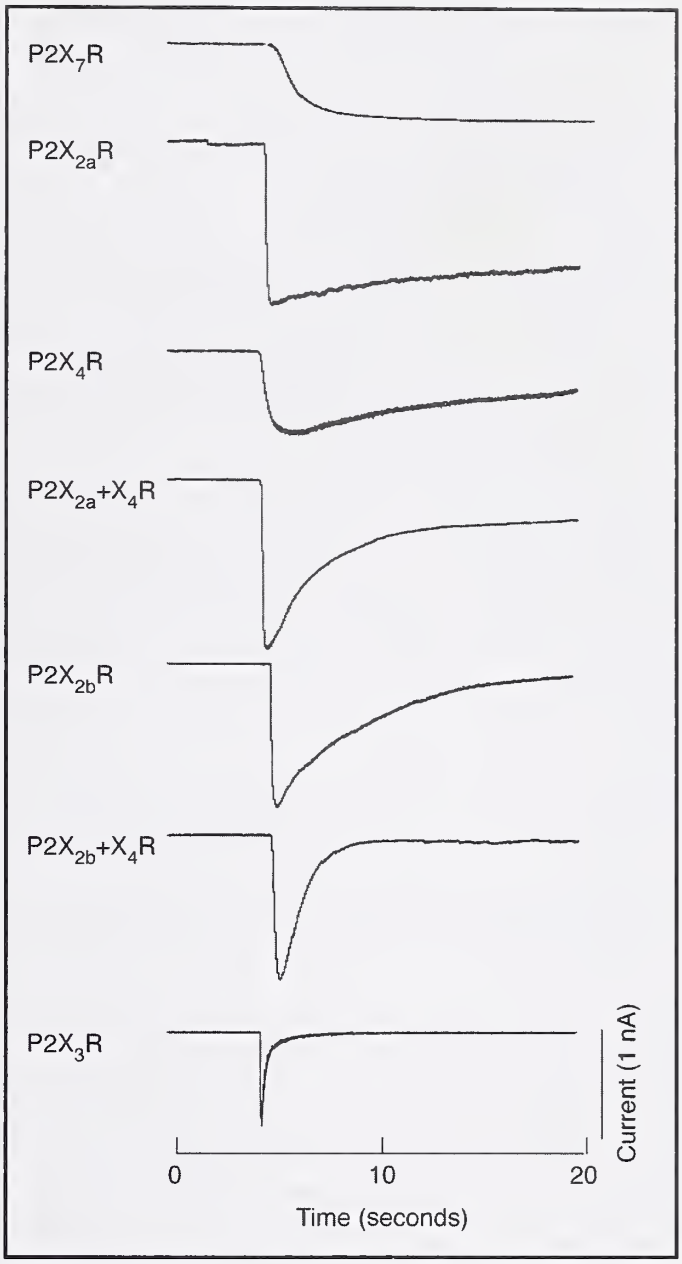

FIGURE 4.2

The receptor-specific pattern of ATP-induced current signals in HEK293 cells expressing extracellular nucleotide-activated purinergic receptors (P2XRs). The horizontal bar indicates the exposure time to 100 microM ATP, and the current traces shown are from cells clamped at –60 mV.

The structural bases of P2XR ligand preferences and channel kinetics have been incompletely characterized. We tested the hypothesis that the affinity of agonists for binding domain accounts for a ligand-specific desensitization pattern. We generated chimeras using receptors with sensitivity to ATP that varied in the following order: $P2X_4R > P2X_{2a}R = P2X_{2b}R >> P2X_7R$. Chimeras with the ectodomain Ile^{66}-Tyr^{310} sequence of $P2X_2R$ and the Val^{61}-Phe^{313} sequence of $P2X_7R$ in the backbone of $P2X_4R$ were expressed but formed nonfunctioning channels. $P2X_{2a}{+}X_4R$ and $P2X_{2b}{+}X_4R$ chimeras with the Val^{66}-Tyr^{315} ectodomain sequence of $P2X_4R$ in the backbones of $P2X_{2a}R$ and $P2X_{2b}R$ were functional and exhibited increased sensitivity to ligands compared with both parental receptors. The chimeras also desensitized faster than parental receptors and in a non–ligand-specific manner. However, like parental $P2X_{2b}R$ and $P2X_{2a}R$, chimeric $P2X_{2b}{+}X_4R$ desensitized more rapidly than $P2X_{2a}{+}X_4R$, and the rate of desensitization of $P2X_{2a}{+}X_4R$ increased by substituting its Arg^{371}-Pro^{376} intracellular C-terminal sequence with the Glu^{376}-Gly^{381} sequence of $P2X_4R$. The results indicate the relevance of interaction between ectodomain and flanking regions around the transmembrane domains on ligand potency and receptor activation. Furthermore, the ligand potency positively correlated with the rates of receptor desensitization but did not affect the C-terminal–specific pattern of desensitization. We currently focus on crystallization and modeling of the ectodomain of $P2X_4R$.

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Cyclic nucleotides and calcium signaling and secretion

Andric, Gonzales-Iglesias, Kostic,[a] He, Tomic, Stojilkovic

The interrelationship between spontaneous and receptor-controlled electrical activity and cyclic nucleotide signaling has not been clarified in pituitary cells. Our results indicate that the cells fire action potentials spontaneously and that the associated calcium influx is sufficient to trigger PRL release, whereas removal of extracellular calcium abolishes pacemaking activity and basal PRL release. Spontaneous calcium transients and PRL release were also abolished by removal of extracellular sodium, but were not affected by tetrodotoxin, a blocker of voltage-gated sodium channels. In addition, nickel, a blocker of T-type calcium channels and cyclic nucleotide-gated cation (CNG) channels, inhibited spontaneous calcium transients and PRL release. Consistent with the role of CNG channels in pacemaking, high calcium and magnesium inhibited calcium transients and PRL release. Furthermore, activation of adenylyl cyclase by forskolin and inhibition of phosphodiesterases by 3-isobutyl-1 methylxanthine (IBMX) initiated calcium transients in quiescent lactotrophs and increased the frequency of spiking and PRL release. Inhibition of protein kinase A did not affect the stimulatory actions of forskolin and IBMX on calcium signaling and PRL release. The mRNAs encoding the alpha subunits of RCNG1, OCNG1, and CCNG1 of channels were detected in mixed pituitary cells by RT-PCR, whereas GH3 immortalized pituitary cells expressed only RCNG1. The messages for the hyperpolarization-activated CNG channel (HCN) isoforms were also detected in pituitary and GH3 cells, with HCN3 and HCN4 subtypes expressed abundantly. Our current investigations focus on electrophysiological characterization of CNG and HCN channels in lactotrophs, gonadotrophs, and somatotrophs.

In addition to forskolin, cholera toxin and several G_s protein–coupled receptors stimulated calcium signaling and increased cAMP/cGMP levels, whereas $G_{i/o}$ protein–coupled receptors inhibited spontaneous calcium transients and decreased cAMP/cGMP levels. The stimulatory action of adenylyl cyclase activators on calcium signaling and the parallelism in the changes of cAMP and cGMP levels is consistent with the literature's reports that calcium stimulates nitric oxide (NO) synthase activity. Furthermore, we showed the expression of endothelial and neuronal NO synthases in pituitary cells as well as the expression of NO-sensitive soluble guanlylyl cyclase (sGC). Surprisingly, we found that the abolition of calcium signaling did not block receptor-controlled up- and down-regulation of cyclic nucleotide accumulation, suggesting that cAMP production affects cGMP accumulation. Inhibition of NO synthase activity abolished basal and stimulated cGMP production, but the rise in NO levels above basal was not essential for agonist- and forskolin-induced increase in cGMP production. Adenylyl cyclase–dependent cGMP production was mimicked by expression of a constitutive active catalytic subunit of protein kinase A and accompanied by phosphorylation of the native and recombinant alpha-1-sGC subunit. Inhibition of the phosphorylation by a protein kinase A inhibitor and a dominant negative mutant of regulatory protein kinase A subunit abolished adenylyl cyclase–dependent cGMP production without affecting basal and NO-donor stimulated cGMP production. The results indicate that phosphorylation of the alpha-1 subunit of sGC by protein kinase A enhances the NO-dependent sGC activity, most likely by stabilizing the NO/sGC complex. Our current investigations focus on identification of residues that are phosphorylated by protein kinase A.

The coupling between the NO-cGMP signaling pathway and PRL release in pituitary lactotrophs has been previously established. However, the messenger that mediates the action of this signaling pathway on hormone secretion and the affected calcium-dependent or -independent secretory mechanism have not yet been identified. Inhibition of constitutively expressed neuronal NO synthase decreased NO and cGMP levels and increased basal PRL release. The addition of a slowly releasable NO donor increased cGMP levels and inhibited basal PRL release in a time-dependent manner. Expression of inducible NO synthase also increased NO and cGMP levels and inhibited basal, depolarization-induced, and TRH-induced PRL release, whereas inhibition of the enzyme decreased NO and cGMP production and recovered PRL release. None of these treatments affected spontaneous and stimulated voltage-gated calcium influx. At basal NO levels, the addition of permeable cGMP analogs did not inhibit PRL secretion. At elevated NO levels, inhibition of cGMP production and facilitation of its degradation did not reverse inhibited PRL secretion. These experiments indicate that NO inhibits calcium-dependent PRL secretion in a cGMP-independent manner and downstream of voltage-gated calcium influx.

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Receptor-controlled calcium mobilization and secretion

Tomic, Andric, Zivadinovic,[b] *Van Goor,*[c] *Stojilkovic; in collaboration with Basta*

Several G protein–coupled receptors expressed in lactotrophs stimulate or inhibit spontaneous voltage-gated calcium influx and PRL secretion. Here we focus on two calcium-mobilizing receptors: thyrotropin-releasing hormone (TRH) and endothelin-A (ET_A). The TRH receptor is expressed in lactotrophs and GH immortalized cells and is coupled to the phospholipase C signaling pathway through $G_{q/11}$ protein. Activation of the receptors leads to the rapid increase in calcium mobilization from intracellular stores and PRL release accompanied by sustained facilitation of calcium influx and secretion. Whereas the calcium-mobilizing action of TRH receptors is well defined, the mechanism of facilitation of sustained calcium influx and PRL secretion has not been clarified. Consistent with the hypothesis that spontaneously active K_{ir} and/or erg channels participate in the control of pacemaking in lactotrophs, we observed the stimulatory effects of cesium on cytosolic calcium in a fraction of cells. Others have shown the expression of mRNA for cesium-sensitive K_{ir}1.0 and 2.0 subfamilies and cesium-sensitive erg channels in rat pituitary and immortalized GH_3 cells. Furthermore, it has been suggested that the stimulatory action of calcium-mobilizing TRH agonist on calcium influx in lactotrophs occurs through partial inhibition of K_{ir} and/or erg channels. In our experiments, blockers of these channels did not affect calcium signaling and PRL release. A blocker of M channels was also ineffective in inhibiting TRH-induced biphasic calcium response. The lack of effects of these blockers on TRH-induced calcium signaling and secretion does not argue against the agonist action on currents observed by others but rather indicates that the currents are not critical for generating the agonist-specific bidirectional and biphasic patterns of signaling and secretion.

Like TRH, the ET_A receptor also induces rapid calcium release from intracellular stores and PRL secretion but, in contrast to TRH, sustained activation of ET_A receptor leads to inhibition of voltage-gated calcium influx and PRL secretion. Both the activation of adenylyl cyclase by forskolin and the addition of cell-permeable 8Br-cAMP stimulated PRL secretion but did not affect the ET-1–induced sustained inhibition of voltage-gated calcium influx, suggesting that the cAMP–protein kinase A signaling pathway does not mediate the inhibitory action of ET-1 on calcium influx. However, the addition of ET-1, an ET_A receptor agonist, induced depolarization of cells and enhancement of calcium influx upon calcium mobilization in lactotrophs treated overnight with pertussis toxin. Consistent with the role of pertussis toxin–sensitive potassium channels in ET-1–induced hyperpolarization of controls but not in pertussis toxin–treated cells, ET-1 decreased the cell input resistance and activated cesium-sensitive potassium current. On the other hand, apamin and paxilline, specific blockers of calcium-activated SK- and BK-type potassium channels, respectively, E-4031, a blocker of the *ether a-go-go* potassium channel, and linopirdine, a blocker of the M-type potassium channel, did not affect the agonist-specific patterns of calcium signaling and PRL secretion. The results suggest that ET-1 inhibits voltage-gated calcium influx through activation of cesium-sensitive channels, presumably the $G_{i/o}$-controlled inward rectifier potassium channels, and that the agonist also inhibits PRL release, but downstream of calcium influx. Currently, we are analyzing the effects of ET-1 on PRL release in pertussis toxin–treated cells.

In collaboration with Milan Basta, we also examined the relevance of high-dose intravenous immunoglobulin (IVIG) on calcium signaling by scavenging complement fragments C3b and C4b in order to test the hypothesis that exogenous immunoglobulin molecules also bind to the anaphylatoxins C3a and C5a and thereby neutralize their proinflammatory effects. Single-cell calcium measurements in human HMC-1 mast cells showed that a rise in intracellular calcium caused by C3a and C5a is inhibited in a concentration-dependent manner with IVIG, $F(ab)'_2$-IVIG, and nonrelated human monoclonal antibody. Exogenous immunoglobins also suppressed C3a/C5a-induced thromboxane generation and histamine release from HMC-1 cells and whole-blood basophils. In a mouse model of asthma, immunoglobulin treatment reduced cellular migration to the lung. Lethal C5a-mediated circulatory collapse in pigs was prevented by pretreatment with $F(ab)'_2$-IVIG. Molecular modeling, surface plasmon resonance, and Western blot analyses suggest physical association between anaphylatoxins and the constant region of $F(ab)'_2$. Such binding could interfere with the role of C3a and C5a in inflammation.

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THE BIOTRANSFORMATION OF ENDOBIOTICS BY SULFONATION

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We aim to gain insight into the molecular mechanisms and biologic implications of modifying substances by sulfonation, a fundamental process in the biotransformation of endobiotics as well as of drugs and xenobiotics. Sulfonation, the transfer of an SO_3^- group from the universal donor molecule 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to an acceptor molecule, is essential for normal growth and development and for maintenance of the internal milieu. Sulfonated macromolecules such as glycosaminoglycans and proteoglycans are involved in cell surface and connective tissue structures and bone formation. Sulfolipids are concentrated in the brain, peripheral nerves, and reproductive tissues. Sulfonation of tyrosine residues is a widespread posttranslational modification of many secretory and membrane proteins. Furthermore, glycoprotein hormones are modified by the sulfonation of specific saccharide moieties, creating unique structural motifs, with important functional consequences. Sulfonation is also of major importance in the biotransformation of low molecular weight compounds such as catecholamines, iodothyronines, neuroendocrine peptides, cholesterol, and the cholesterol metabolites oxysterols, bile acids, vitamin D, and steroid hormones. By modulating availability of the biologically active steroid/sterol form, sulfotransferases can influence biologic activity and thus play an essential role in specific physiologic systems and associated disorders.

Human hydroxysteroid sulfotransferases SULT2A1, SULT2B1a, and SULT2B1b

Fuda, Lee, Higashi, Yanai, Javitt, Strott

Often referred to as hydroxysteroid sulfotransferases, the enzymes that sulfoconjugate neutral steroids constitute the SULT2 family, which consists of two subfamilies, SULT2A1 and SULT2B1. Whereas the SULT2A1 subfamily consists of a single form, the SULT2B1 subfamily consists of the two isoforms SULT2B1a and SULT2B1b, which derive from an alternative exon 1. We have cloned, overexpressed, and purified all human SULT2 subfamily members for biochemical, functional, and structural analyses. SULT2A1 is commonly referred to as dehydroepiandrosterone (DHEA) sulfotransferase because DHEA is considered the preferred substrate, although the isozyme has a broad substrate specificity. It would seem that such labeling is arbitrary as SULT2A1, depending on the species, can have an equal or greater affinity for other neutral steroids, e.g., pregnenolone. In contrast to SULT2A1, the SULT2B1 isoforms sulfonate DHEA less efficiently and have a much narrower substrate preference. Importantly, the SULT2B1 isoforms are structurally distinct from SULT2A1 as well as from all other known cognate cytosolic sulfotransferases. Furthermore, the SULT2B1 isoforms, which differ only at their amino-terminal ends, exhibit a more defined substrate selectivity, e.g., SULT2B1a avidly sulfonates pregnenolone but poorly sulfonates cholesterol, whereas SULT2B1b functions as a cholesterol sulfotransferase. It is notable that the substrate specificity demonstrated by the SULT2B1 isoforms is in part dependent on the uniqueness of their amino-terminal ends. The fact that the SULT2A1 and SULT2B1 isozymes are differentially expressed and display dissimilar substrate specificities strongly suggests that they play distinct biologic roles.

The cloning of the human *SULT2B1* gene, a significant advance in the area of steroid/sterol sulfotransferases, has led to the finding that the SULT2B1b subtype acts as a specific cholesterol sulfotransferase. It is noteworthy that cholesterol sulfate is a widely distributed sulfolipid and, in human plasma, is quantitatively more significant than any other known sterol sulfate. Although knowledge of the physiologic role(s) of cholesterol sulfate is still evolving, the steroid has been implicated in a number of biologic systems. To date, the clearest and most investigated physiologic role for cholesterol sulfate is in keratinocyte differentiation and development of the barrier. It has also been implicated in adrenal and gonadal steroid synthesis, in the regulation of cholesterol and fatty acid synthesis, in controlling sperm capacitation, in the regulation of serine proteases, and in the induction of platelet aggregation. The fact that cholesterol sulfate circulates in blood and its emerging role as a regulatory molecule suggest that it might be categorized as a hormone.

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Crystal structure of human cholesterol sulfotransferase (SULT2B1b)

Fuda, Lee, Strott; in collaboration with Pedersen

We have determined the crystal structure of SULT2B1a and SULT2B1b bound to the substrate donor product 3′-phospoadenosine-5′-phosphate (PAP) at 2.9 Å and 2.4 Å, respectively, as well as that of SULT2B1b in the presence of the acceptor substrate pregnenolone at 2.3 Å. These structures reveal a different catalytic binding orientation for the substrate from the previously determined structure for prototypical hydroxysteroid sulfotransferase (SULT2A1) bound to DHEA. In addition, the amino-terminal helix, comprising residues D19 to K26, which determines the specificity difference between the SULT2B1 isoforms, becomes ordered as pregnenolone binding covers the substrate-binding pocket.

We previously suggested that the specificity difference for cholesterol and pregnenolone between the SULT2B1a and SULT2B1b isoforms could be traced to the unique amino-terminal residues 19-DISEI-23. Results from alanine scanning mutagenesis of the 19-DISEI-23 region revealed that only the I20A and I23A mutants knocked out cholesterol sulfonating activity. We then showed that this activity could be partially restored by conservative substitutions such as leucine and methionine. I20 and I23 lie on the same side of the helix facing on the inside of the hydrophobic pocket, whereas residues S21 and E22 are exposed to solvent. Interestingly, the residues corresponding to 19-DISEI-23 of SULT2B1b are 4-PPPFH-8 in SULT2B1a. With three prolines in a row, it is unlikely that the residues are able to form an alpha-helix and therefore would be unable to cover the opening to the substrate binding pocket as seen in the SULT2B1b structure. Although we do not yet have a crystal structure of SULT2B1b with cholesterol bound, the position of pregnenolone is such that the O20 ketone is only 3.4 Å from atom CD1 of I20 and the C21 atom is 6.0 Å from atom CD1 of I23. Thus, the residues may be in positions to form positive van der Waal's interactions with the longer side chain of the C17 atom of cholesterol. Slight conformational changes of the protein, however, may be necessary to accommodate the longer side chain of cholesterol.

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Expression of cholesterol sulfotransferase (SULT2B1b) in human skin and primary cultures of human keratinocytes

Higashi, Fuda, Yanai, Lee, Strott; in collaboration with Kanzaki

Although its physiologic significance remains unclear, cholesterol sulfate is present in relatively high concentrations in the epidermis of human skin, particularly in the granular layer. We examined expression of the gene encoding cholesterol sulfotransferase (SULT2B1b) and found, consistent with the involvement of cholesterol sulfate in keratinocyte development, progressive expression of SULT2B1b mRNA in primary cultures of normal human epidermal keratinocytes (NHEK) during Ca^{2+}-induced differentiation. The rise in SULT2B1b mRNA expression was associated with parallel increases in the expression of protein and enzymatic activity. Of the three known steroid/sterol sulfotransferase isozymes, only SULT2B1b was expressed in normal human skin and cultured NHEK, as would be expected if the sole purpose of SULT2 expression in skin were the production of cholesterol sulfate, given that SULT2B1b functions as a selective cholesterol sulfotransferase. The expression of SULT2B1b in skin was localized to the granular layer of the living epidermis, in contrast to the barrier protein involucrin, an early marker of keratinocyte differentiation strongly expressed in the immediate suprabasal spinous layer. The intense expression of SULT2B1b in the granular layer of the epidermis is consistent with the layer containing the highest concentration of epidermal cholesterol sulfate.

A variety of regulatory effects have been ascribed to cholesterol sulfate in the epidermis, such as regulation of transcription, protein kinase C, and lipid metabolism, suggesting multifunctional actions for this molecule. To further our understanding of the role of cholesterol sulfate, we conducted experiments to determine when and where during keratinocyte development the sulfotransferase producing cholesterol sulfate is expressed. Immunocytochemical analysis revealed that SULT2B1b was localized to the granular layer of the epidermis, suggesting that it is a late marker of keratinocyte differentiation. If SULT2B1b were an early marker of keratinocyte differentiation, it would be expected to have an expression pattern similar to that of involucrin, which was clearly expressed by cells in the immediate suprabasal region or spinous layer, i.e., the earliest cells in the pathway toward keratinocyte differentiation. However, SULT2B1b had an expression pattern that was the same as that of another barrier protein filaggrin, an acknowledged late marker of differentiation. The epidermis is a perpetually renewing tissue whereby keratinocytes arise from stem cells in the basal layer and move through a series of cellular differentiation events until, as dead squames, they are finally sloughed off from the outer stratum corneum. Cholesterol sulfate might play a significant role in the proper functioning of the stratum corneum and the normal sloughing of dead cells, a process termed desquamation. For instance, cholesterol sulfate is able to retard desquamation by inhibiting serine proteases in the stratum corneum that are responsible for the degradation of cell adhesion structures known as desmosomes. In addition, cholesterol sulfate can influence cell cohesiveness by affecting the stability of corneocyte lipid bilayers. The inhibitory influence of cholesterol sulfate on normal desquamation, which presumably occurs maximally in the lower stratum corneum would subsequently be removed by the presence of a cholesterol sulfate sulfohydrolase in the outer stratum corneum, thus allowing normal desquamation to occur. This hypothesis is supported by the finding of markedly elevated cholesterol sulfate in the stratum corneum of patients with recessive X-linked ichthyosis, a scaling disorder that is a form of abnormal desquamation resulting from a deficiency in stratum corneum steroid sulfatase activity.

Almost 90 percent of the cholesterol sulfate formed during Ca^{2+}-induction of keratinocyte differentiation was found in the cellular membrane fraction, with only 10 percent in the soluble cell fraction. This finding, along with previous reports of cholesterol sulfate localization to membranes of erythrocytes and spermatozoa, suggests that cellular membrane localization of cholesterol sulfate might be a general phenomenon. It has been suggested that cholesterol sulfate is involved in membrane structure and stability, as indicated by its ability to protect red blood cells against osmotic lysis or fusion with animal viruses. We are currently trying to identify what NHEK cellular membranes are involved in the incorporation of cholesterol sulfate.

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Expression of cholesterol sulfotransferase (SULT2Bb) in human platelets

Yanai, Javitt, Higashi, Fuda, Strott

Among the many regulatory actions of cholesterol sulfate is its ability to influence blood clotting and fibrinolysis. In addition, cholesterol sulfate has been shown to support platelet aggregation. We have documented the presence of the enzyme (SULT2B1b) that sulfonates cholesterol in human platelets and examined the influence of plasma lipoproteins on the expression and activity of this enzyme. We detected SULT2B1b mRNA by RT-PCR and found it to be the only steroid/sterol sulfotransferase expressed in these discoid anucleate particles. Using real-time PCR for quantification, we found that, at 4°C, the level of SULT2B1b mRNA in platelets was maintained but markedly diminished over a period of four hours at 37°C. The loss of SULT2B1b mRNA, however, was significantly reduced in the presence of HDL but not LDL. The stabilizing influence of HDL was attributable specifically to its apolipoprotein A-I (apoA-I) component, whereas apolipoprotein A-II (apoA-II) and apolipoprotein E (apoE) were without effect. Importantly, there was a direct correlation between platelet SULT2B1b mRNA and protein levels in the presence or absence of lipoprotein that was reflected in enzymatic activity and cholesterol sulfate production. An exciting recent finding is that platelet membranes contain a specific and saturable cholesterol sulfate-binding activity. We are currently embarking on the purification, characterization, and cloning of this binding factor.

Mouse ortholog of the human *SULT2B1* gene

Fuda, Yanei, Strott; in collaboration with Shimizu

We have cloned a novel mouse hydroxysteroid sulfotransferase cDNA and determined the organization of its gene structure. The new mouse sulfotransferase (SULT2B1a) and its closely related isoform (SULT2B1b) are derived from a single gene as a result of an alternative exon 1 and differential splicing. Thus, the only structural distinction between the SULT2B1 isoforms lies in their amino-terminal ends. The mouse *Sult2B1* gene structure is identical to that of the human *SULT2B1* gene, indicating that the gene and its products are highly conserved. Furthermore, the mouse SULT2B1 isoforms, similar to human SULT2B1, have a predilection for cholesterol and pregnenolone over DHEA, which is ordinarily sulfonated by the SULT2A1 subfamily.

Differential expression of mouse SULT2B1a, SULT2B1b, and SULT2A1, along with their distinct substrate preferences, has physiologic relevance. For instance, the exclusive expression of SULT2B1a in the central nervous system and its preference for pregnenolone as a substrate would be in keeping with the importance of pregnenolone sulfate as a neurosteroid involved in learning and memory processes. The sole expression of SULT2B1b in skin and the fact that it clearly has a predilection for cholesterol as a substrate are in keeping with the importance of cholesterol sulfate as a regulatory molecule in keratinocyte differentiation and development of the epidermal barrier. On the other hand, the overwhelming expression of SULT2A1 in the liver would be in keeping with its role in general metabolism involving both xenobiotics and endobiotics. Interestingly, the mouse *Sult2B1* and *Sult2A1* genes are differentially expressed during embryonic development, with the former expressed at all stages from E8.5-E19 and the latter not expressed until E19. The results suggest that expression of the mouse *Sult2B1* gene is more significant during early development than the *Sult2A1* gene, especially the expression of SULT2B1a during the early stages of brain development and the expression of SULT2B1b during growth of epithelia.

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We study the molecular genetics and pathogenesis of heritable metabolic human disorders, currently focusing on type I glycogen storage diseases (GSD-I). GSD-Is are caused by deficiencies in the endoplasmic reticulum (ER)–bound glucose-6-phosphatase (G6Pase) system, which comprises G6Pase and glucose-6-phosphate transporter (G6PT). G6PT translocates glucose-6-phosphate (G6P), the product of gluconeogenesis and glycogenolysis, from cytoplasm to the lumen of the ER while G6Pase hydrolyzes the intraluminal G6P to glucose and phosphate. Together, G6Pase and G6PT maintain glucose homeostasis. Deficiencies in G6Pase and G6PT cause GSD-1a and GSD-1b, respectively. Both manifest the symptoms of G6Pase deficiency as characterized by growth retardation, hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia. GSD-Ib patients also present with the unique symptoms of neutropenia and myeloid dysfunction, symptoms not obviously related to G6P metabolism in the gluconeogenic tissues. Over the last 20 years, dietary therapy has alleviated some metabolic abnormalities of GSD-I patients and delayed disease progress. However, the underlying disease remains untreatable and the efficacy of dietary treatment is frequently limited by poor compliance. Therefore, long-term complications still develop in adult patients and can be rectified only with an understanding of the molecular genetics and pathogenesis of GSD-1.

Structure-function study of G6PT

Chen, Pan, Shieh, Chou

The G6PT is a 10-transmembrane–domain ER protein. To date, 69 G6PT mutations, including 28 missense mutations and two codon-deletions, have been identified in GSD-Ib patients. Using a pSVL-based expression assay, we previously characterized 15 of the missense mutations and one codon-deletion mutation. A lack of sensitivity in the assay limited discrimination between mutations that lead to loss of function and mutations that leave low residual activity. Based on an adenoviral vector-mediated expression system, we recently reported an improved G6PT assay and its use in the functional characterization of all 30 codon mutations found in GSD-Ib patients. Twenty of the naturally occurring mutations completely abolish microsomal G6P uptake activity while the other 10 mutations, including five previously characterized ones, partially inactivate the transporter. This information should greatly facilitate genotype-phenotype correlation. We also reported a structure-function analysis of G6PT. In addition to the three destabilizing mutations reported earlier, we showed that the G50R, C176R, V235del, G339C, and G339D mutations also compromise G6PT stability. Mutation analysis of the amino-terminal domain of G6PT shows that it is required for optimal G6P uptake activity. Finally, we showed that degradation of both wild-type and mutant G6PT is inhibited by a potent proteasome inhibitor, lactacystin, demonstrating that G6PT is a substrate for proteasome-mediated degradation.

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The signature motif in G6PT

Pan, Chen, Mansfield, Chou; in collaboration with Salani, Varesio

Amino acid sequence alignments have identified a signature motif shared by G6PT and a family of transporters of phosphorylated metabolites. Two null signature motif mutations have been identified in the *G6PT* gene of GSD-Ib patients. We are now characterizing the activity of seven additional mutants within the motif. Five mutants lack microsomal G6P uptake activity and one retains residual activity, suggesting that the signature motif in G6PT is a functional element required for microsomal glucose-6-phosphate transport.

Pan C-J, Chen L-Y, Mansfield BC, Salani B, Varesio L, Chou JY. The signature motif in human glucose-6-phosphate transporter is essential for microsomal transport of glucose-6-phosphate. *Hum Genet* 2003;112: 430-433.

A murine model of GSG-Ib

Chen, Shieh, Lin, Pan, Mansfield, Chou; in collaboration with Gao, Lee, Moses, Roe, Ward, Westphal*

In addition to disrupted glucose homeostasis, GSD-Ib patients, in response to the bacterial peptide f-Met-Leu-Phe, have unexplained and unexpected defects in neutrophil respiratory burst, chemotaxis, and calcium flux as well as intermittent neutropenia. We generated a *G6PT* knockout (*G6PT*$^{-/-}$) mouse that mimics all known defects of the human disorder and used the model to further our understanding of the pathogenesis of GSD-Ib. We demonstrate that the neutropenia is caused directly by the loss of G6PT activity; that chemotaxis and calcium flux, induced by the chemokines KC and macrophage inflammatory protein-2, are defective in *G6PT*$^{-/-}$ neutrophils; and that local production of these chemokines and the resultant neutrophil trafficking *in vivo* are depressed in *G6PT*$^{-/-}$ ascites during an inflammatory response. The bone and spleen development of *G6PT*$^{-/-}$ mice is delayed and accompanied by marked hypocellularity of the bone marrow, elevation of myeloid progenitor cell frequencies in both organs, and a corresponding dramatic increase in granulocyte colony–stimulating factor levels in both GSD-Ib mice and humans. Thus, in addition to transient neutropenia, a sustained defect in neutrophil trafficking due to both the resistance of neutrophils to chemotactic factors and reduced local production of neutrophil-specific chemokines at sites of inflammation may underlie the myeloid deficiency in GSD-Ib. These findings demonstrate that G6PT is not just a G6P transport protein but also an important immunomodulatory protein, whose activities need to be addressed in treating the myeloid complications in GSD-Ib patients.

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Gene therapy for GSD-Ia

Ghosh, Sun, Chou; in collaboration with Byrne

G6Pase is a highly hydrophobic protein anchored to the ER by nine-transmembrane helices. The protein cannot be expressed in a soluble form and must embed correctly in the ER membrane and couple with other proteins to be functional. Therefore, enzyme replacement therapy is not an option, but somatic gene therapy, targeting G6Pase to the liver and kidney, is an attractive possibility. Using a G6Pase knockout (*G6Pase*$^{-/-}$) mouse model generated in our laboratory, we have shown that a combined adeno (Ad-mG6Pase) and adeno-associated virus (AAV) serotype 2 (AAV2-mG6Pase)–mediated gene transfer leads to sustained G6Pase expression in both the liver and kidney and corrects the murine GSD-Ia disease for at least 12 months.

The most dangerous clinical presentation in GSD-Ia is the life-threatening hypoglycemia. Because AAV2-mG6Pase-mediated transgene expression increases slowly, typically peaking five to seven weeks postinfusion, we coinfused AAV2-mG6Pase with Ad-mG6Pase, which allows the infused animals to survive weaning. We now demonstrate that neonatal *G6Pase*$^{-/-}$ mice infused only with AAV1-mG6Pase, a recombinant AAV of serotype 1, not only survived weaning but also lived to 12 months of age. Our results suggest that human GSD-Ia would be treatable by gene therapy.

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We conduct both basic and clinical investigations to understand molecular mechanisms of inflammatory/autoimmune and neurodegenerative diseases and to develop novel approaches for the treatment of these diseases. Our studies focus on understanding the regulation and physiological functions of several genes and their products, including uteroglobin (UG), soluble phospholipases A_2 ($sPLA_{2s}$), palmitoyl-protein thioesterase (PPT), and neutral ceramidase. UG is a multifunctional secreted protein with potent $sPLA_2$-inhibitory and immunomodulatory activities. We have demonstrated that UG-deficient mice suffer from IgA-nephropathy, the most common primary renal glomerular disease, for which there is no effective treatment, and are studying the molecular mechanisms of the disease to develop novel therapeutic approaches. We have also isolated another gene encoding PPT and found that inactivating mutations in this gene lead to infantile neuronal ceroid lipofuscinosis (INCL), the most severe form of childhood Batten disease, for which there is no effective treatment, and are conducting a pilot study to determine whether Cystagon™ may be effective treatment for INCL. We are using a mouse model of INCL to understand the molecular mechanisms of the disease and to develop novel therapeutic approaches using gene transfer and embryonic stem (ES) cell technologies.

Novel role of uteroglobin as an innate repressor of PGD_2 receptor–mediated *COX-2* gene expression: implications for allergic asthma

Mandal,[b] *Zhang, Chowdhury, Ray, Mukherjee; in collaboration with Pattabiraman*

Uteroglobin is secreted by the tracheobronchial epithelia of virtually all mammals. We previously reported that ovalbumin challenge in uteroglobin knock-out mice elicits an exaggerated allergic response characterized by increased expression of Th2 cytokines, eotaxin, and eosinophil infiltration in the lungs. We report here that levels of prostaglandin D_2 are markedly elevated in the bronchoalveolar lavage fluid of these mice and that the animals produce high levels of cyclooxygenases-2, a critical enzyme for the production of prostaglandins, which are abrogated by uteroglobin pretreatment. Given that prostaglandin D_2 mediates allergic airway inflammation via its receptor DP and that the molecular mechanism of DP signaling is unknown, we studied DP signaling *in vitro* and determined the role of uteroglobin in this process. We found that DP signaling is mediated via mitogen-activated protein kinases (P38 and p42/44) and protein kinase C in a cell-specific manner leading to the activation of nuclear factor (NF)-kappaB, which stimulates cyclooxygenase-2 expression. Most important, we found that uteroglobin blocks DP signaling, inhibits NF-kappaB activation, and, consequently, suppresses cyclooxygenase-2 gene expression. We propose that uteroglobin is a critical component of an innate homeostatic mechanism against inadvertent stimulation of allergen-induced inflammatory response in the airways.

Chowdhury B, Mantile-Selvaggi G, Miele L, Cordella-Miele E, Zhang Z, Mukherjee AB. Lys 43 and Asp 46 in alpha-helix 3 of uteroglobin are essential for its phospholipase A_2 inhibitory activity. *Biochem Biophys Res Commun* 2002;295:877-883.

Inhibition by uteroglobin of prostaglandin F_{2alpha} receptor–mediated activation of NF-kappaB and *COX-2* gene expression

Mandal,[b] Ray, Zhang, Chowdhury, Mukherjee

Via its F_{2alpha} receptor (FP), prostaglandin F_{2alpha} regulates many important physiological processes such as inflammation, uterine smooth muscle contraction, and parturition. It has been suggested that such effects of PGF_{2alpha} may be mediated by the production of pro-inflammatory lipid mediators. However, the molecular mechanism by which FP signaling regulates the production of these mediators remained unclear until now. We report here that FP signaling is mediated by PKC and that it activates NF-kappaB, which up-regulates cyclooxygenase-2. Cyclooxygenase-2 is a mediator-inducible enzyme that is critical for the production of mediators from arachidonic acid, which is released from cellular phospholipids by cytosolic phospholipase A_2, the expression of which is also stimulated by PGF_{2alpha}. Most important, uteroglobin inhibits FP-mediated NF-kappaB activation and cyclooxygenase-2 expression. We propose that uteroglobin is an essential component of a novel innate mechanism of homeostasis against inadvertent activation of FP-mediated stimulation of cyclooxygenases-2 expression and prevents the generation of pro-inflammatory lipid mediators.

Wang CY, Lei HJ, Huang CY, Zhang Z, Mukherjee AB, Yuan CJ. Induction of cyclooxygenase-2 by staurosporine through the activation of nuclear factor for IL-6 (NF-IL6) and activator protein 2 (AP2) in an osteoblast-like cell line. *Biochem Pharmacol* 2002;64:177-184.

CC10-null mice are predisposed to lung tumorigenesis by NNK in cigarette smoke

Zhang, Mukherjee; in collaboration with Linnoila, Yang

Ninety percent of all human lung cancers are related to cigarette smoking. Both tobacco smoke and lung tumorigenesis are associated with reduced levels of Clara cell 10 kD protein (CC10), a multifunctional, steroid-inducible, secreted protein that is naturally present in the respiratory system of virtually all mammals. CC10 expression is rarely detectable in human non–small cell cancers, despite being abundantly produced by progenitors of normal airway epithelial cells. Its expression is drastically reduced following exposure of animals to the methylnitrosamine NNK, the main carcinogen in tobacco smoke, and during SV40-induced carcinogenesis. Moreover, induced expression of CC10 in cancer cells reverses the transformed phenotype. Here we report that, compared with wild-type controls, *CC10*-knock-out mice exposed to NNK are highly susceptible (30 percent knock-out versus 5 percent wild type) to dramatically increased proliferation of airway epithelial cells and development of adenoma of the lung within five to 12 months of NNK exposure. Interestingly, the hyperproliferation and tumorigenesis in NNK-treated knock-out mice were associated with activation of *Ras* and the mitogen-activated protein kinase, and 50 percent of the adenomas had the *K-Ras* mutation. We propose that CC10 deficiency leads to increased susceptibility to carcinogen-induced lung cancers, raising the possibility that this protein possesses tumor suppressor activity.

Interferon-gamma stimulates the expression of a novel secretoglobin gene in lymphoblasts

Choi, Zhang, Mukherjee

Interferons (IFN) are a family of cytokines that alert the immune system to viral infections of host cells. The IFNs (IFN-alpha, IFN-beta, and IFN-gamma) interact with specific cellular receptors and stimulate the production of second messengers, leading to the expression of antiviral and immune-modulatory genes. It has been previously reported that IFN-gamma stimulates the expression of uteroglobin (UG), the founding member of the *secretoglobin* superfamily, with immunomodulatory properties. We demonstrate here that, in addition to UG, IFN-gamma stimulates a novel secretoglobin gene in lymphoblasts that bears 74 percent sequence identity with lipophilin-B. A BLAST search also revealed that the gene is highly similar to YGB, a lymphocyte-specific secretoglobin. However, unlike YGB, the gene we characterized is expressed not only in lymphoblasts but also in other cell types, including colon carcinoma cells. Moreover, the deduced protein sequence demonstrates that the protein does not contain the C-terminal cysteine residue characteristic of the secretoglobins, including YGB. We named the gene *colon carcinoma and lymphoblast-associated secretoglobin (CCLAS)*. Although the function of the gene is yet to be determined, the stimulation of its expression by IFN-gamma may suggest an important immunological role.

Safety and efficacy of intratracheal recombinant human Clara cell protein in a newborn piglet model of acute lung injury

Pilon-Clayton; in collaboration with Chandra, Chester, Davis, Drexler, Koo, Kowalewska, Levine, Pollack, Welch

Despite the widespread use of exogenous surfactant, acute and chronic lung injury continues to be a major cause of morbidity in preterm infants. CC10 is a protein produced by Clara cells that inhibits phospholipase A2 and has anti-inflammatory and antifibrotic properties. We studied whether intratracheal (IT) recombinant human Clara cell protein (rhCC10) could safely minimize lung injury in a newborn piglet model of acute lung injury. Twenty-nine newborn piglets were given Survanta® and then ventilated for 48 hours receiving the following: room air (group 1); 100 percent O_2 (group 2); or 100 percent O_2 and 25, 5, or 1 mg/kg (groups 3, 4, and 5, respectively) of IT rhCC10. We performed laboratory studies, oxygen ratios, static pressure-volume curves, bronchoalveolar lavage (for inflammatory markers), and histologic analyses over the 48-hour study period. Pulmonary compliance and oxygenation significantly improved in animals receiving 5 mg/kg IT rhCC10 compared with room air and 100 percent O_2 controls. Reductions in inflammatory markers were seen in animals receiving rhCC10, although changes did not reach statistical significance. We noted no significant toxicity. In this newborn piglet model of hyperoxic lung injury, rhCC10 appeared safe and improved pulmonary function and may thus represent a promising therapy for the prevention of lung injury in preterm infants.

Chandra S, Davis JM, Drexler S, Kowalewska J, Koo HC, Chester D, Pollack S, Welch R, Pilon A, Levine CR. Safety and efficacy of intratracheal recombinant human Clara cell protein in a newborn piglet model of acute lung injury. *Pediatr Res* 2003;54:509-515.

Magnetic resonance imaging of the brain of a mouse model of INCL

Zhang, Mukherjee; in collaboration with Hofmann, Koretsky, Munasinghe

Neuronal ceroid lipofuscinoses (NCL) are the most common (1 in 12,500) heritable progressive encephalopathies of children. Inactivating mutations in the PPT gene cause INCL, a heritable lysosomal storage disease. PPT facilitates the degradation of posttranslationally acylated polypeptides by cleaving thioester linkages that connect the lipids with the polypeptides that are acylated. A deficiency of the enzyme leads to abnormal deposition of acylated proteins or peptides (ceroids), causing INCL pathogenesis. Given that the PPT gene is expressed at high levels in the retina and the brain, children afflicted with INCL become blind by two years and brain dead by age four. Recently, a mouse model of INCL was developed by gene targeting, allowing detailed pathophysiologic analyses and the development of novel therapeutic approaches for this disease. To assess the outcome of new treatment modalities, we investigated five age- and sex-matched PPT knock-out mice and five wild-type littermates with a 7-Tesla MRI horizontal scanner operating on a Bruker Avance platform. We synchronized image acquisition with the respiration rate, maintained core body temperature at 37°C, and administered the contrast agent Gd DTPA (0.2 cm^3 /kg) via the tail vein. We acquired fast spin-echo, multislice images in the transverse and sagittal planes to encompass the whole brain.

In addition, we acquired T_1 and T_2 weighted images in five selected slices (1 mm apart) by using, respectively, spin-echo and saturation recovery experiments. T_1 and T_2 maps were generated with an analysis algorithm. Our results indicate that cortical degeneration in PPT-knockout mice could be determined by MRI, paving the way for using this animal model for assessing the outcome of various treatment modalities.

Treatment of INCL patients with cysteamine bitartrate

Levin, Zhang, Mukherjee; in collaboration with Caruso, Fischbeck, Gropman

Given that thioester bonds are susceptible to nucleophilic attack, drugs with nucleophilic properties (e.g., cysteamine bitartrate, trade name Cystagon™) may have therapeutic potential for INCL. Last year, we demonstrated that the drug not only disrupts thioester linkages in S-acylated polypeptides in cultured cells from INCL patients but also mediates the depletion of intracellular ceroid deposits and prevents their reaccumulation. Taken together, the results raised the possibility that Cystagon™ has the potential to be an effective treatment for INCL. Recognizing that INCL is a uniformly fatal disease, for which currently there is no effective treatment, and that Cystagon™ has been in clinical use for more than two decades with a proven record of safety, we have implemented a pilot study to determine whether the drug is beneficial for INCL patients. We have received approval to treat five INCL patients and have so far recruited two patients. Preliminary evaluations indicate stabilization of the retinal functions and cortical degeneration. The most dramatic results were obtained from the electron microscopic analyses of patients' while blood cells. After the initiation of Cystagon™ treatment, the mononuclear cells show virtually no ceroid depositions in their lysosomes compared with the cells before treatment. So far, the patients do not require antiepileptic medications. No adverse effects have been encountered with the treatment. The study will continue until we recruit five patients, and final analyses will be made when all data are available.

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Neutral ceramidase gene: role in preventing ceramide-induced apoptosis

Choi, Anderson,[c] Zhang, Ray, Mukherjee; in collaboration with Popescu

The sphingolipid ceramide, a natural constituent of our daily diet, is a mediator of apoptosis in many cell types. However, the mechanism that prevents ceramide-induced apoptosis is poorly understood. It may involve a nonlysosomal ceramidase in the gastrointestinal tract that manifests an optimal activity in the neutral to alkaline pH range (neutral/alkaline ceramidase), degrading ceramide into sphingosine and free fatty acid. In this study, we first characterized the murine neutral ceramidase gene, mapped its chromosomal location, determined its tissue-specific expression, and delineated its role in ceramide-induced apoptosis both *in vitro* and *in vivo*. Our results show that the gene structure of murine neutral ceramidase (N-CDase) is virtually identical to that of the human N-CDase. It is expressed at a high level in mouse kidneys and in specific segments of the upper gastrointestinal tract. We also found that neutral ceramidase mRNA expression is stimulated by its substrate, C2-ceramide. Most important, we found that mice fed with C2-ceramide or injected with a specific inhibitor of neutral ceramidase before feeding ceramide show dramatically increased apoptosis in mesangial cells *in vitro* and in gastrointestinal tract *in vivo*. Our results raise the possibility that neutral ceramidase is part of a novel homeostatic mechanism to prevent apoptosis in vital organs such as the gastrointestinal tract and the kidneys.

Choi MS, Anderson MA, Zhang Z, Zimonjic DB, Popescu N, Mukherjee AB. Neutral ceramidase gene: role in regulating ceramide-induced apoptosis. *Gene* 2003;315:113-122.

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MOLECULAR BIOLOGY, REGULATION, AND BIOCHEMISTRY OF UDP-GLUCURONOSYLTRANSFERASE ISOZYMES

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Mammalian UDP-glucuronosyltransferase (UGT) isozymes are critical catalysts for detoxifying endogenous metabolites and numerous potentially injurious lipid-soluble phenols derived from the diet and environment. The isozymes detoxify by conjugating glucuronic acid with metabolites, drugs, toxins, and environmental chemicals to make them water-soluble and excretable. Glucuronidation reactions prevent accumulation of neurotoxic levels of plasma bilirubin, inactivate many drugs, and avert the mutagenicity and carcinogenicity of aromatic hydrocarbons such as benzo(a)pyrene found in cigarette smoke and automobile emissions. Moreover, UGTs prevent accumulation of dietary phenols that inhibit enzymes. On the other hand, extensive glucuronidation can be disadvantageous. Thus, the premature clearance, associated with UGT metabolism, of many orally administered therapeutic drugs is a longstanding problem. Metabolism is overcome by administering compensatory higher doses, which can lead to serious side effects, and, for decades, drug inefficiency has been the impetus to developing inhibitor(s) of UGT. The enzymatic mechanism(s) and properties that enable UGTs to convert numerous unrelated lipid-soluble phenols to innocuous glucuronides remain unknown, along with methods to control conjugation and prevent premature clearance of therapeutic drugs. An important research aim, therefore, is to understand the properties and mechanism(s) of this system of detoxification.

Phosphorylation of UGT proteins with signaling

Basu, Mitra, Kole,[a] Kubota[b]

While analyzing UGTs to understand glucuronidation requirements in human colon cell lines, we discovered that the isozymes require phosphorylation that is regulated via signaling. Given that phosphorylation is mediated by certain PKC isozymes or tyrosine kinase(s), the action of classical PKC agonists and antagonists as well as the effects of PKC translocation-specific inhibitor peptides confirmed the involvement of signaling events. Furthermore, immunocomplexing of UGT from solubilized human LS180 colon cells with antiUGT traps PKC isozymes. Parallel hydrogen-peroxide (H_2O_2) stimulation of UGT phosphorylation and glucuronidation activity and inhibition of H_2O_2-enhanced activity by catalase and herbimycin demonstrate that cellular oxidants are signal(s) for the PKC-UGT system. Specifically, our results demonstrate that UGT1A1, 1A7, and 1A10 undergo phosphorylation by PKC(s), which is, in turn, phosphorylated and activated by tyrosine kinase(s). For UGT2B family members that generally metabolize endogenous substrates, we found evidence of direct phosphorylation by tyrosine kinase. In addition to three PKC phosphorylation sites, we showed that UGT2B7 has two tyrosine phosphorylation sites. Alteration of one or all PKC sites in UGT2B7 that generated a triple-mutant had no effect on activity while a single or double mutation at tyrosine sites caused dominant negative activity. The highly specific PKC inhibitor calphostin-C and general kinase inhibitor curcumin inhibited all UGT activities in cell culture. Given that both herbimycin-C and genistein are tyrosine kinase inhibitors, UGT1A1, 1A7, and 1A10 were sensitive to the former, and UGT2B7 was more sensitive to the latter. In summary, our results demonstrate that UGTs undergo phosphorylation and that UGT1A family members are phosphorylated via PKC, with signaling mediated via oxidative stress. At least one UGT2B family member requires tyrosine phosphorylation.

Overexpression of PKCs in conjunction with mutants of UGT1A7 suggested that PKC isozymes phosphorylate specific sites. In addition, evidence derived by comparing normal isozyme-specific peptides with mutant peptides demonstrated that at least three different PKC isozymes can phosphorylate UGT1A7 and 1A10 at positions 73, 202, and 432. Mutations at positions 75 and 112 in UGT1A1 and at positions 73 and 202 in 1A7 and 1A10 were dominant negative while alteration of a third common position, 435 or 432, indicated that the phospho-group(s) plays a unique and novel role in catalysis by operationally controlling pH optima and substrate selection. Alteration of serine-432 to glycine in UGT1A7 caused a dramatic shift in its pH optimum from 8.5 to 6.4 in parallel with the acquisition of new substrate activities or loss of activities resembling substrate selections by 1A10. Substitution of serine-432 in 1A7 with a number of other amino acid residues retained the pH 8.5 optimum seen in the wild-type enzyme. In parallel with *in vitro* activity, cultures of COS-1 cells expressing S432G-UGT1A7, but not wild-type 1A7, avidly glucuronidated the new substrate 17beta-estradiol, similar to wild-type 1A10 and its S432G mutant. For the first time, we presented evidence that phospho-groups play an operational role in catalysis rather than just causing the typical conformational change in protein structure.

Basu NK, Kole L, Owens IS. Evidence for phosphorylation requirement for human bilirubin UDP-glucuronosyltransferase (UGT1A1) activity. *Biochem Biophys Res Commun* 2003;303:98-104.

Location and properties of UGT determine control of gastrointestinal chemical absorption

Basu, Kole;[a] in collaboration with McDonagh

Using Northern blot, *in situ*, and biochemical analyses, we determined distribution and function of the isozymes UGT1A1, the bilirubin-metabolizing isozyme, and UGT1A7, 1A8, 1A9, and 1A10 and found them to be segmentally distributed throughout the GI in mucosal epithelia. Recombinant isozymes exhibited a pH optimum of 6.4 or pH 7.6 and/or broad ranges; activity was either unaffected or progressively inhibited by increasing substrate concentrations. All exhibited wide substrate selections for dietary and environmental chemicals but with differing optimal conditions. We demonstrated the impact of glucuronidation on drug and chemical absorption at the GI level by exploiting our recent finding that UGTs require phosphorylation. [^{33}P]Orthophosphate incorporation into immunoprecipitable UGTs confirmed phosphorylation. The simultaneous suppression of UGT radiolabeling and activity in cell lines by the general kinase inhibitor curcumin and the PKC-specific inhibitor calphostin-C also confirmed the requirement for phosphorylation. Using curcumin-treated mouse duodenal loops, we demonstrated that free bilirubin (a marker) uptake was dramatically increased concurrently with decreases in bilirubin-glucuronides in lumen, tissue, and portal blood. The results represent the first direct evidence that UGTs control GI absorption of polyphenols and that maintenance of their phosphorylation is required to limit chemical absorption.

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Transient inhibition of gastrointestinal UGT activity markedly improves mycophenolic acid immunosuppressant activity

Basu, Kole,[a] Chowdhury

While the promising immunosuppressant mycophenolic acid (MPA) is being widely prescribed for both adult and child renal transplant patients and for autoimmune diseases, serious side effects are associated with high dosage requirements because of extensive glucuronidation of the drug. We found that the cellular distribution and biochemical properties of the primary metabolizers of MPA UGT1A7, 1A8, 1A9, and 1A10 contribute significantly to high oral dose requirements. *In situ* hybridization studies revealed that UGT1A7-, 1A9-, and 1A10-mRNAs are located in GI mucosal epithelia; studies with microsomes isolated from adjacent specimens showed that esophagus, ileum, duodenum, and colon have moderately high to high glucuronidating activities. Recombinant UGTs avidly glucuronidate MPA, showing nearly linear increases in activity with concentration (up to 800 microM); only UGT1A9 showed typical saturation kinetics. Each isozyme generates about 80 percent ether-linked and about 20 percent acylglucuronide. To establish the *in vivo* impact of MPA glucuronidation, we used the general kinase inhibitor curcumin and highly specific protein kinase C inhibitor calphostin-C on LS180 colon cells to inhibit UGT's newly discovered phosphorylation requirement. Transient inhibition of glucuronidation by oral pretreatment with curcumin before MPA administration caused a six-fold greater immunosuppression of antigen-stimulated spleen cytotoxic T-lymphocyte proliferation in mice. Hence, glucuronidation can adversely impact drug efficacy. Moreover, curcumin pretreatment to inhibit GI-distributed UGTs represents a model for increasing bioavailability of highly glucuronidated drugs.

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We study the molecular, biochemical, and cellular processes that underlie dysmorphic syndromes and birth defects, with a current focus on the inborn errors of cholesterol synthesis, including Smith-Lemli-Opitz syndrome.

Smith-Lemli-Opitz syndrome

Correa-Cerro, Cozma, Krakowiak, Sterner, Tierney, Wassif, Wright, Porter; in collaboration with Javitt, Kelley, Shackleton

The Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive, multiple malformation syndrome characterized by dysmorphic facial features, mental retardation, hypotonia, poor growth, and variable structural anomalies of the heart, lungs, brain, limbs, gastrointestinal track, and genitalia. The SLOS phenotype is extremely variable. At the severe end of the phenotypic spectrum, infants often die due to multiple major malformations. In contrast, at the mild end of the phenotypic spectrum, SLOS combines minor physical malformations with behavioral and learning problems. The syndrome is attributable to an inborn error of cholesterol biosynthesis that blocks the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol. Our laboratory initially cloned the human 3beta-hydroxysterol delta^7-reductase gene (*DHCR7*) and demonstrated mutations of the gene in SLOS patients. To date, we have identified 91 mutations of *DHCR7*. In support of our clinical protocol Clinical and Basic Investigations into Smith-Lemli-Opitz syndrome, we have genotyped over 50 SLOS patients and have continued to identify novel mutations of the gene. To further our understanding of the mechanisms underlying the broad phenotypic spectrum in this human malformation syndrome, we have use deuterium oxide labeling to measure residual DHCR7 activity in fibroblasts from patients with known genotypes and well-characterized phenotypes.

The most common SLOS mutation, IVS8-1G→C, is a single nucleotide G to C change at the minus one position of the splice acceptor in the eighth intron. Aberrant splicing to a cryptic splice acceptor results in the insertion of 134 base pairs of intronic sequence into the mRNA. The mutation results in an allele with no enzymatic function and accounts for about one-third of the identified mutant alleles. The second most common SLOS mutation is T93M. Other common mutations include W151X, V326L, R404C, and R352W. SLOS may be more common than typically thought. The carrier frequency for the IVS8-1G→C allele is on the order of 1 percent in Caucasians. Surprisingly, although few patients of African heritage have been identified, the carrier frequency for this allele in African Americans was found to be 0.73 percent. These carrier frequencies predict a minimum disease incidence for SLOS of at least 1/40,000 and 1/75,000 in Caucasians and African Americans, respectively.

In addition to basic science work aimed at understanding the pathophysiological processes underling SLOS, we initiated a clinical protocol to study genotype/phenotype correlations and endocrinological, neurological, dental, speech therapy, and behavioral aspects of SLOS. Notably, the SLOS behavioral phenotype includes autistic features. Therapy for SLOS includes dietary cholesterol supplementation. Currently, we have enrolled over 50 SLOS patients and have initiated a new protocol to study the safety and efficacy of simvastatin therapy in SLOS.

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Mouse models of Smith-Lemli-Opitz syndrome

Correa-Cerro, Wassif, Porter; in collaboration with Beachy, Cooper, Kelley, Lu

Using gene targeting in murine embryonic stem cells, we have produced three SLOS mouse models, namely, a null mutation and two hypomorphic point mutations. Similar to human patients, mouse pups that are homozygous for the null mutation have variable craniofacial anomalies, are growth retarded, feed poorly, and appear weak. The homozygous null (*Dhcr7*$^{-/-}$) pups die during the first day of life due to failure to feed. Biochemical characterization showed that the mutant pups had markedly elevated serum and tissue 7-DHC levels as well as reduced serum and tissue cholesterol levels. Cleft palate was present in 9 percent of the *Dhcr7*$^{-/-}$ pups and is found in approximately one-third of all SLOS patients. To characterize further the neurological abnormalities seen in the mutant mouse pups, we measured the response of cortical neurons to the

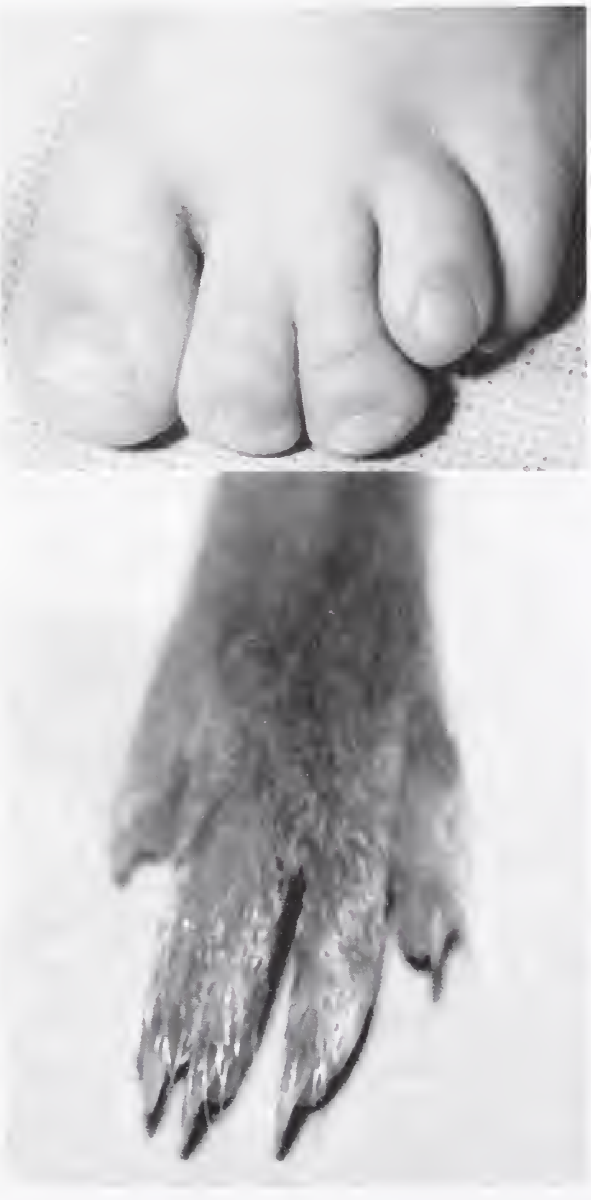

FIGURE 5.1

Figure 5.1 Legend: Second and third toe syndactyly in mouse and human Smith-Lemli-Opitz syndrome (SLOS)
The SLOS is an autosomal recessive human mental retardation/ malformation syndrome attributable to an inborn error of cholesterol biosynthesis. The syndrome is caused by mutations of the 7-dehydrocholesterol reductase gene. The most common physical finding in SLOS is syndactyly of the second and third toes. A viable mouse model of SLOS was produced by "knocking-in" a T93M missense mutation into the 7-dehydrocholesterol reductase gene. The phenotype of SLOS mice with the T93M mutation includes second and third toe syndactyly.

neurotransmitters GABA and glutamate. Comparing mutant with control neurons, we observed no significant difference when the response to GABA was measured. However, the glutamate response of mutant neurons was significantly decreased compared with the response observed in control cortical neurons. A decreased glutamate response is consistent with the phenotypic observation of poor feeding by the mutant animals. Glutamate receptors are involved in neuronal pattern formation, long-term potentiation and depression, memory acquisition, and learning. Neurological dysfunctions, including poor feeding, hypotonia, mental retardation, and behavioral problems, are major clinical problems in SLOS. The impaired glutamate response observed in the mouse model may yield insight into the etiology of some of the neurological dysfunction seen in SLOS. In collaboration with Bai Lu's laboratory, we are characterizing synapse formation in neurons derived from these mice.

Given that the *Dhcr7*$^{-/-}$ pups die during the first day of life, we are not able to study postnatal brain development, myelination, and behavior or to test therapeutic interventions. For this reason, we have developed two missense alleles (*Dhcr7*$^{\text{T93M}}$ and *Dhcr7*$^{\text{L99P}}$) and a conditional *Dhcr7* mutant allele (*Dhcr7* $^{\text{loxPdelta3-5loxP}}$). The T93M mutation is the second most common mutation found in human patients. *Dhcr7*$^{\text{T93M/T93M}}$ and *Dhcr7*$^{\text{T93M/delta3-5}}$ are viable. Biochemically, the mice have SLOS with a gradient of biochemical severity (*Dhcr7*$^{\text{delta3-5/delta3-5}}$> *Dhcr7*$^{\text{T93M/delta3-5}}$> *Dhcr7*$^{\text{T93M/T93M}}$). Characterization of these hypomorphic SLOS mice is in progress and includes phenotypic, histological, biochemical, and behavioral analysis.

We are using *Dhcr7*$^{\text{T93M/delta3-5}}$ mice to test the efficacy of therapeutic interventions on tissue sterol profiles. Initial results with cholesterol therapy show improvement of the sterol defect in peripheral tissues. Initial results using simvastatin indicate that we can ameliorate the biochemical defect in both peripheral and brain tissue. We plan to study the effect of these therapeutic interventions on neuromuscular/behavioral abnormalities.

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Wassif CA, Zhu P, Kratz L, Krakowiak PA, Battaile KP, Weight FF, Grinberg A, Steiner RD, Nwokoro NA, Kelley RI, Stewart RR, Porter FD. Biochemical, phenotypic, and neurophysiological characterization of a genetic mouse model of RSH/Smith-Lemli-Opitz syndrome. *Hum Mol Gen* 2001;10:555-564.

Lathosterolosis

Cozma, Krakowiak, Wassif, Porter

Lathosterol 5-desaturase catalyzes the conversion of lathosterol to 7-dehydrocholesterol in the enzymatic step immediately preceding the defect in SLOS. Thus, to further our understanding of the roles of decreased cholesterol versus increased 7-dehydrocholesterol in the SLOS, we disrupted the mouse lathosterol 5-desaturase gene (*Sc5d*) by using targeted homologous recombination in embryonic stem cells. The *Sc5d*$^{-/-}$ pups are stillborn and have micrognathia and cleft palates and limb patterning defects. Many of the malformations found in the mutant mice resemble malformations found in SLOS and are consistent with impaired hedgehog signaling during development. Biochemically, the mice have markedly elevated serum and tissue lathosterol levels and decreased cholesterol levels.

One goal of producing a lathosterolosis mouse model was to identify a phenotype corresponding to the human malformation syndrome. We have now identified a human patient (infant) with lathosterolosis, a human malformation syndrome not previously described. Fibroblasts from this patient show decreased cholesterol and increased lathosterol levels. Mutation analysis showed that the patient is homozygous for a single A to C nucleotide change at position 137 in *SC5D,* resulting in a mutant enzyme in which the amino acid serine substitutes for tyrosine at position 46. Both parents were heterozygous for the mutation. Phenotypically, the infant resembled severe SLOS. Malformations found in both the human patient and the mouse model include growth failure, abnormal nasal structure, abnormal palate, micrognathia, and postaxial polydactyly. A unique feature of lathosterolosis is the clinical finding of mucolipidosis in the affected infant. This clinical presentation is not reported in SLOS and may help in clinically separating the two disorders. This lysosomal storage disorder can be replicated in embryonic fibroblasts from the *Sc5d* mutant mouse model.

Krakowiak PA, Wassif CA, Kratz L, Cozma D, Kovářová M, Harris G, Grinberg A, Yang Y, Hunter AGW, Tsokos M, Kelley RI, Porter FD. Lathosterolosis: an inborn error of human and murine cholesterol synthesis due to lathosterol 5-desaturase deficiency. *Hum Mol Genet* 2003;12:1631-1641.

Characterization of LIM homeobox genes *Lhx2* and *Lhx9*

Wassif, Porter; in collaboration with Westphal, Yang

Lhx2 and *Lhx9* are two closely related LIM homeobox genes that are essential for the development of multiple organ systems. *Lhx2* mutant mice are anophthalmic, have forebrain malformations, and die *in utero* due to inefficient definitive erythropoiesis. Lhx2 also functions to pattern the dorsal telencephalon. *Lhx9* has an overlapping but distinct expression pattern compared with *Lhx2*. *Lhx9* mutant mice are agonadal; thus, Lhx9 is essential for gonad development. Both *Lhx2* and *Lhx9* are expressed in the developing limb; however, neither *Lhx2* nor *Lhx9* mutant mice have limb defects. In collaboration with both the Westphal and Yang laboratories, we are analyzing *Lhx2/Lhx9* compound mutants to determine the combined functions of these two genes. Double *Lhx2* and *Lhx9* mutant embryos have limb truncation defects. Thus, these two LIM homeobox genes are functionally redundant with respect to limb development. Further characterization of the role of *Lhx2/Lhx9* in limb development is in progress.

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PROTEIN-MEDIATED REMODELING OF MEMBRANE LIPID BILAYERS

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Membrane remodeling is an important stage of diverse cell biological processes, including exo- and endocytosis, fertilization, membrane trafficking, and different stages of enveloped virus infection. We hypothesize that specialized proteins mediate the remodeling by promoting the changes in the membrane shape that both prime the membranes for the bilayer rearrangement and drive the remodeling to completion. More specifically, we hypothesize that lateral interactions between fusion proteins drive membrane rearrangements from local hemifusion (the merger of contacting membrane monolayers) to an opening of an expanding fusion pore. In our recent work on the pathway of lipid bilayer fusion, we have theoretically analyzed the possible mechanisms of the transition between stalk, a local hemifusion intermediate, and pore. We have also focused on the properties of the hemifusion stage in the prototype fusion reaction driven by influenza virus hemagglutinin (HA) and found that hemifusion is generated by fusion machinery that is distinct and independent from the machinery that forms expanding fusion pores. Finally, we have studied the mechanisms by which short cationic peptides remodel cell membranes to enter into cytoplasm without degradation. Our data indicate that these cell-penetrating peptides are internalized by an endocytic pathway.

Theoretical analysis of formation, structure, and decay of the hemifusion diaphragm

Chernomordik; in collaboration with Kozlov, Kozlovsky

While widely considered to be a key stage in membrane fusion, hemifusion is poorly understood both on the structural level and in terms of the physical forces involved. In our recent work, we analyzed theoretically the pathways of the intermediate membrane structures emerging in the course of hemifusion and the forces driving evolution of the intermediates into a fusion pore. This evolution could proceed by one of the following routes. The stalk-pore pathway suggests that the stalk expands radially and brings the distal monolayers of the two membranes together into a single bilayer. Opening of a fusion pore within the hemifusion diaphragm (HD) completes the fusion reaction. To allow formation of the pore rim, the HD radius has to exceed a certain value approximately equal to the lipid monolayer thickness. In the second model, the fusion pore forms directly from the stalk and constitutes from the very beginning a bilayer connection between the membranes.

We show that a fusion stalk spontaneously expands into an HD only if the contacting monolayers of the fusing membranes consist of lipids such as dioleoyl phosphatidylethanolamine or its mixtures with other lipids. For a "bilayer" lipid, such as dioleoyl phosphatidylcholine, stalk expansion requires an external force. In the case of biological fusion, the force might be generated by the fusion proteins that pull apart the diaphragm rim. To address the mechanism of fusion pore formation, we analyzed the distribution of the lateral tension emerging in the HD in response to the establishment of lateral equilibrium between the deformed and relaxed portions of lipid monolayers. We showed that the tension concentrates along the HD rim and reaches values sufficiently high to rupture the bilayer and form the fusion pore. The prediction of a crack-like pore propagating along the HD rim readily explains an earlier observation of the lack of movement of aqueous dyes while total fusion pore conductance increases, as has has been observed for HA-mediated fusion. In general, our analysis supports the hypothesis that transition from a hemifusion to a fusion pore involves radial expansion of the stalk.

Kozlovsky Y, Chernomordik LV, Kozlov MM. Lipid intermediates in membrane fusion: formation, structure, and decay of hemifusion diaphragm. *Biophys J* 2002;83:2634-2651.

Kinetic differentiation of influenza hemagglutinin fusion and hemifusion machines

Mittal, Leikina, Chernomordik; in collaboration with Bentz

Membrane fusion mediated by influenza virus hemagglutinin (HA) yields different phenotypes depending on the surface density of activated HAs. A crucial question is whether different phenotypes arise from different fusion machines or whether different numbers of identical fusion machines yield different probabilistic outcomes. If fusion were simply a less probable event than hemifusion and required a larger number of identical fusion machines to occur first, then we could make two predictions. First, because more machines are present, fusion should have a shorter average delay time than hemifusion. Second, fusion should have a longer execution time of lipid mixing after it begins than hemifusion; obviously, the full event cannot occur more rapidly than the partial event.

To compare fusion machines that yield different fusion phenotypes, we developed a novel, simple, and rigorous computer-driven kinetic analysis of video fluorescence microscopy of fusion between HA-expressing cells and fluorescently labeled erythrocytes. The assay provides quantitative information about the waiting time for initiation of dye spread and the time required for completion of dye spread for a large number of individually fusing pairs.

Using this technique, we screened many individual HA-expressing cells fusing with erythrocytes and identified cell pairs with either full or partial redistribution of fluorescent lipids. Only the full lipid mixing phenotype represented fusion with contents mixing. Partial lipid mixing corresponds to an unrestricted hemifusion phenotype (lipid mixing without content mixing) in which the lipidic connection between the bilayers dissociates before full redistribution of lipid dye. The new technique allowed us to test the above predictions. Given (1) that we observed no correlation between the waiting times for the onset of lipid mixing for fusion and the unrestricted hemifusion phenotypes and (2) that the execution time for fusion was faster than that for hemifusion, we concluded that distinct and independent machines generate the two fusion phenotypes.

Mittal A, Leikina E, Chernomordik L, Bentz VJ. Kinetically differentiating influenza hemagglutinin fusion and hemifusion machines. *Biophys J* 2003;85:1-12.

Cell-penetrating peptides: a re-evaluation of the mechanism of cellular uptake

Melikov, Ramos, Chernomordik; in collaboration with Gait, Lebleu, Richard, Verbeure, Vives

During the last decade, several proteins such as HIV-1 Tat, *Drosophila* Antennapedia homeoprotein, and HSV-1 VP22 have been shown to traverse the cell membrane by a process called protein transduction and to reach the nucleus while retaining biological activity. Short cationic peptides derived from protein-transduction domains that are responsible for the cellular uptake of these proteins (cell-penetrating peptides or CPP) can be internalized in most cell types and, more important, allow the cellular delivery of conjugated (or fused) biomolecules. Based largely on CPP translocation studies that relied on fluorescence microscopy of fixed cells and fluorescence-activated (FACS) analysis, the literature has ascribed the internalization of CPP to a mechanism that does not involve endocytosis.

In our recent work, we have demonstrated that cell fixation, even in mild conditions, leads to the artefactual uptake of CPP peptides. Moreover, the peptides bind strongly to the cell plasma membrane and remain associated with cells even after repeated washings. As a result, flow cytometry analysis is not a valid method for evaluating cellular uptake unless the protocol includes a step of trypsin digestion of the cell membrane–adsorbed peptide.

Fluorescence microscopy on live unfixed cells shows characteristic endosomal distribution of peptides. Flow cytometry analysis indicates that the kinetics of uptake are similar to the kinetics of endocytosis. Peptide uptake is inhibited by incubation at low temperature and cellular ATP pool depletion. Similar data obtained for Tat-conjugated peptide nucleic acids indicate that the cellular internalization of CPP proceeds by an endocytic pathway. Future work will clarify the specific mechanisms by which CPP promotes remodeling of membranes to facilitate either endocytosis of the peptide-conjugated cargo and/or its rapid escape from endosome into the cytoplasm and nucleus of living cells to avoid degradation.

Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, Lebleu B. Cell-penetrating peptides: a re-evaluation of the mechanism of cellular uptake. *J Biol Chem* 2003;278:585-590.

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ADVANCES IN THE METHODOLOGY OF ELECTROPHORETIC SEPARATION

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Our work aims at increasing the effectiveness of electrophoretic separations of biological macromolecules and subcellular particle-sized species by application of separation theory through development of computer programs and by instrumental and procedural optimization at a level that is practical for the vast majority of biochemical laboratories. We continued to work on improving the method of "direct electroelution" of proteins from polyacrylamide gels and that of "static noncovalent" coating of capillaries to reduce to a negligible value electroosmotic flow in capillary electrophoresis (CE). Using rat synaptic vesicles as a model, we have been attempting to develop a novel approach to deciphering protein complexes based on their cross-linking and fractionation by capillary electrophoresis. The complexes are to be continuously blotted to membrane, identified as SNARE protein complexes, and then followed by a mass-spectrometric (MS) characterization of interacting proteins. We have also been attempting to improve the resolution at low gel concentration of fluorescently labeled saccharides by using acetate as the trailing constituent of a disc electrophoresis buffer system and to replace 2D-PAGE in proteomic analysis of a representative membrane protein by a three-stage procedure of SDS-PAGE, electroelution, IPG-IEF, and band transfer into MS.

Discontinuous buffer systems operative at pH 2.5 to 11, 0°C and 25°C made available on the Internet

Chrambach; in collaboration with Wheeler

During the past year, our section made available on the Internet, for the first time, buffer systems across the entire pH scale capable of concentrating a sample and enhancing resolution (http://www.ncbi.nlm.nih.gov/Class/wheeler/jovin.html); previously, these systems could be accessed only by mainframe computer, on microfiche and magnetic tapes.

Wheeler D, Chrambach A, Ashburn P, Jovin TM. Discontinuous buffer systems operative at pH 2.5 to 11, 0 and 25°C, available on the Internet. *Electrophoresis*; in press.

Hydrophobic protein transfer from IEF to MS

Buzás, Chrambach

A second advance initiated during the past year concerns the frequent inapplicability of 2-dimensional electrophoresis in the proteomics of membrane proteins, which constitute about one-half of all cellular proteins. That inapplicability derives from the insolubility in detergent solutions of hydrophobic proteins attached to lipids once they are separated from SDS. Such separation with replacement of SDS by neutral or amphoteric detergents is a prerequisite to isoelectric focusing (IEF), the first dimension of 2-D PAGE in conventional proteomic analysis. Accordingly, we are attempting to circumvent the insolubility of membrane proteins on IEF by conducting SDS-PAGE as a first dimension, directly electroeluting the SDS-proteins of interest as described (Radko *et al.*, 2002), and applying the electroeluted proteins to IEF in the most effectively solubilizing medium presently available, a mixture of weak detergent and chaotrope. Given that the electroeluate concentration of SDS is very low and that lipid moieties are likely to be removed during SDS-PAGE, that three-step procedure is likely to make membrane proteins amenable to proteomic analysis. Furthermore, the procedure allows for simultaneous IEF analysis at a number of narrow pI-range immobilized pH gradient (IPG) gels, replacing the intolerably laborious and sample-consuming present practice of multiple 2-D PAGE analysis with first-dimensional IPGs of several narrow pI ranges.

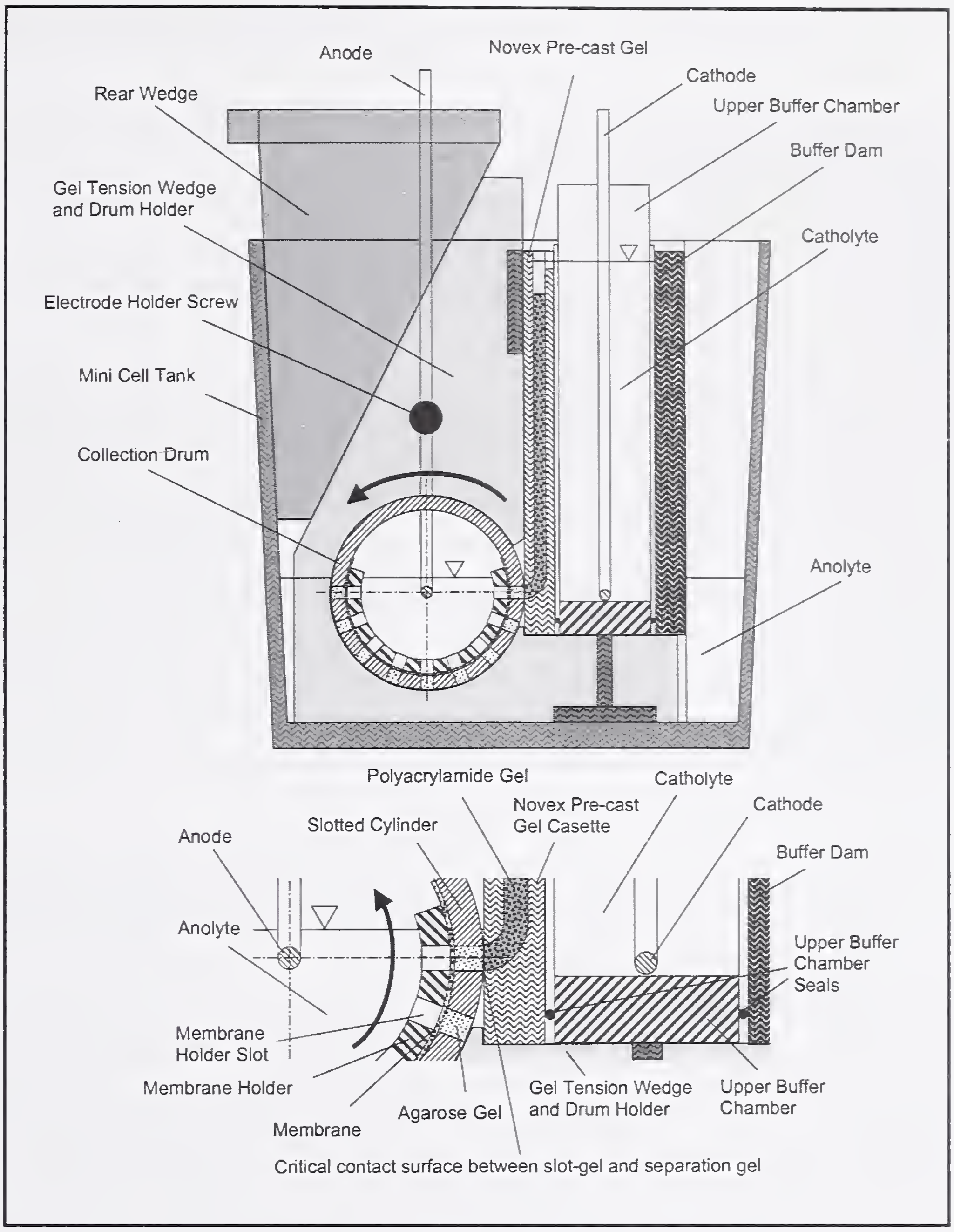

FIGURE 6.1

Schematic drawing of the electroelution device

Size dependence of microparticle transfer from CE to MS

Chrambach; in collaboration with Radko

Using rat synaptic vesicles in lieu of the previously applied scarce sea urchin egg cortical membrane vesicles, our third advance during the year concerns the continuing search for fusogenic proteins involved in addition to the SNARE proteins in vesicle-vesicle fusion. We are attempting to identify such fusogenic membrane proteins by immunodetection of their linked SNARE proteins after chemical cross-linking and then separating them by capillary zone electrophoresis and characterizing them mass spectrometrically after severance of the cross-links, SDS-PAGE, and electroelution.

Sequential multiple protein transfer from PAGE to MS

Antal, Buzás, Chrambach

We designed, constructed, and successfully applied an apparatus for sequential multiple protein band elution and collection on soluble agarose blocks prior to MS (Figure 6.1).

Antal J, Buzás Z, Gilligan JJ, Yergey AL, Chrambach A. Sequential transfer of bands separated on Novex gels into a mass spectrometer. *Electrophoresis*; in press.

Electrophoretic separation of fluorescently labeled glucose oligomers

Buzás, Chrambach; in collaboration with Berna, Cabanes-Macheteau, Taverna

Replacement of the conventional catholyte Tris-glycine with the more rapidly migrating catholyte Tris-acetate allows us to separate glucose oligomers, which, under conventional conditions, are stacked into a single band because the highly charged oligomers are unstacked behind the trailing constituent.

Cabanes-Macheteau M, Chrambach A, Taverna M, Buzás Z, Berna P. Resolution of ANTS labeled glucose oligomers in PAGE at low gel concentration. *Electrophoresis*; in press.

A capillary electrophoresis procedure with improved sensitivity and speed

Chrambach; in collaboration with Chang, Chiu, Huang, Lin

We developed a CE procedure providing increased sensitivity and speed by applying SDS to the background electrolyte at one-fifth to one-third of the injection volume before sample (SYPRO-red–labeled protein) injection. To concentrate the sample, PEG added to the anolyte migrates by electro-osmosis counter to the migration of the protein. Separation of five model proteins by the procedure required 11 minutes with detection limits of 30 pM. The procedure was validated by analysis of casein (0.45 mM) in milk.

Chiu TC, Lin YW, Huang CC, Chrambach A, Chang HT. A simple rapid and sensitive method for analysis of SYPRO red labeled SDS protein complexes by capillary electrophoresis with laser induced fluorescence. *Electrophoresis* 2003;24:1730-1736.

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MICROBIAL PATHOGENS IN HUMAN LYMPHOID TISSUE

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The critical events of many human infectious diseases occur in tissues, in which cells contact neighboring cells and are immersed in a rich heterogeneous microenvironment. Microbes, particularly viruses, may cause disease by disrupting cell-cell interactions. We study the pathogenesis of microbes, specifically HIV-1, in a system of human lymphoid tissue *ex vivo*. The main targets of HIV-1 are activated $CD4^+$ T lymphocytes; *in vivo*, however, nonactivated cells are also infected. The same phenomenon is reproduced in lymphoid tissue *ex vivo*, thus providing a unique opportunity for experimental study. Our first project addressed this phenomenon. In addition, a system of *ex vivo* human lymphoid tissue provides an opportunity to study, under controlled experimental conditions, mechanisms of another hallmark of HIV disease, T cell depletion. Here, we investigated whether uninfected "bystander" $CD4^+$ T cells are depleted in HIV-1–infected tissues. We continue to study the pathogenesis of HIV-1 copathogens in the context of human tissues *ex vivo*. Our third project aimed to understand the pathogenesis of human herpesvirus 6 (HHV-6), which *ex vivo* suppresses HIV-1 variants that use the CCR5 coreceptor but enhances the replication of HIV-1 variants that use CXCR4.

Viral production and depletion of activated and nonactivated cells in HIV-infected tissues *ex vivo*

Biancotto, Margolis, Grivel; in collaboration with Hirsch

Activation is believed to determine the level of cell susceptibility to productive HIV infection. In tissues *in vivo*, however, nonactivated cells are infected as well. Largely unknown are the patterns of T cell activation that render cells susceptible to productive HIV infection, the contribution of nonactivated cells to the viral load, and how lymphocyte activation status determines T cell depletion in lymphoid tissues. We used the system of human lymphoid tissue infected *ex vivo* with HIV-1, developed earlier in our laboratory, to study these issues.

First, we investigated whether there is a correlation between the efficiency of viral production in tissue blocks and the number of activated $CD4^+$ T cells. Viral replication in the tissue becomes noticeable six days after inoculation, and the virus quickly spreads through the tissue thereafter. Analysis of the activation markers CD69, CD25, and HLA-DR revealed that at this time the numbers of cells expressing the "early" activation marker CD69 dramatically decreased, whereas the numbers of T cells expressing the "late" activation markers CD25 and HLA-DR remained at approximately the same level. Despite a strong linear correlation between the level of HIV-1 production and the number of $CD25^+/HLA\text{-}DR^+/CD4^+$ T cells ($r=0.76$ and $p=0.0047$), there is no significant correlation between viral load and expression of CD69 at the time of HIV-1 inoculation and during the few days that follow. Next, we investigated whether T cells expressing late activation markers are the main viral producers. Flow cytometry revealed that both HLA-DR$^+$/CD25$^+$ cells and nonactivated cells (negative for these markers) were productively infected. Thus, unlike in other *in vitro* systems, nonactivated cells in human lymphoid tissue *ex vivo* are capable of supporting HIV-1 productive infection in human lymphoid tissue in parallel to that observed *in vivo*. Nevertheless, we found that an activated T cell is only twice as likely as a nonactivated T cell to be productively infected ($p= 0.005$). However, the contribution of the latter pool to total viral production seems to be small in spite of its larger size. Thus, the number of cells expressing late activation markers in *ex vivo* tissue is a determinant of viral load.

Like infection *in vivo,* productive HIV infection in *ex vivo* tissue results in depletion of CD4$^+$ T cells. We studied the relation between the activation status of an infected cell and its fate. Given that most T cell death caused by HIV occurs through the apoptotic pathway, we monitored the coexpression of an early apoptotic marker and found that infection with HIV-1 selectively increased the expression of this apoptotic marker in activated (CD25$^+$/HLA-DR$^+$) T cells. HIV-1 infection itself may affect the activation status of the cells in tissue, as the proportion of activated cells increases in the productively infected T cell subset. However, the presence of infected T cells did not change the pattern of activation marker expression in uninfected T cells. Thus, infection does not affect the activation status of bystander T cells.

In conclusion, the number of T cells expressing late but not early activation markers at the time that HIV-1 spreads through the tissue determines the efficiency of viral production, indicating that susceptibility of T cells to HIV-1 infection is associated with a particular activation status. Both activated and nonactivated T cells support productive HIV infection. The frequency of productively infected T cells in the latter pool is only half of that in the pool of activated T cells. However, in spite of their abundance, productively infected nonactivated CD4$^+$ T cells do not significantly contribute to total viral production of HIV-1–infected tissue because of their low productivity. Nonactivated HIV-infected T cells therefore constitute, in our *ex vivo* system, a reservoir of longer-living cells slowly releasing virus. Changing the activation status of productively infected T cells alone may modulate viral load. Our results indicate that neither productive infection alone nor activation alone is sufficient to draw a T cell into apoptosis. It appears that only a combination of productive infection and activation is sufficient for T cell apoptosis. Therefore, general immunostimulation may be a critical factor for CD4$^+$ T cell depletion in HIV-infected individuals.

Gondois-Rey F, Grivel J-C, Biancotto A, Pion M, Vigne R, Margolis L, Hirsch I. Segregation of R5 and X4 HIV-1 variants to memory T cell subsets differentially expressing CD62L in *ex vivo* infected human lymphoid tissue. *AIDS* 2002;16:1245-1249.

Grivel J-C, Biancotto A, Ito Y, Gomes R, Margolis LB. Bystander CD4 T lymphocytes survive in HIV-infected human lymphoid tissue. *AIDS Res Hum Retroviruses* 2003;19:211-216.

Bystander CD4 T lymphocytes survive in HIV-infected human lymphoid tissue

Grivel, Biancotto, Ito, Margolis

The mechanisms of depletion of CD4$^+$ T cells in HIV-1–infected tissues are not yet understood. In particular, it remains controversial whether and to what extent uninfected ("bystander") CD4$^+$ T cells die in HIV-infected individuals. We addressed this issue by using the system of human lymphoid tissue *ex vivo*. To determine whether the presence of HIV-1 virions is sufficient to induce a massive death of CD4$^+$ T cells in the absence of productive infection, we adopted the following experimental strategy: we inoculated tissue with infectious HIV-1 and, after productive tissue infection was established, we added nevirapine, a well-known non-nucleotide reverse transcriptase inhibitor, to protect uninfected CD4$^+$ T cells from subsequent productive infection. Because nevirapine is a reverse transcriptase inhibitor, it does not prevent cells that are already productively infected from continuing to produce virus. Thus, in tissues treated according to our protocol, one pool of CD4$^+$ T cells was infected before nevirapine application and continued to produce virus, and the other pool of CD4$^+$ T cells resided in the same tissue and remained uninfected. The CD4$^+$ T cells residing in HIV-infected tissue are by definition "bystanders."

First, we found that, at concentrations equal to or higher than 0.5 microM, nevirapine completely blocks HIV-1 infection *de novo*. In our next experiments, we applied 5-microM nevirapine either at the time of HIV-1 inoculation or on day six following HIV-1 inoculation. We replaced nevirapine at each medium change to maintain its level. When nevirapine was added on day six postinfection, it inhibited *de novo* infection, but HIV-1 continued to replicate in tissue cells infected before nevirapine action. We compared the levels of depletion of CD4$^+$ T cells in control and nevirapine-treated tissues infected with HIV-1. The decrease of CD4$^+$ T cell numbers in infected tissue (not treated with nevirapine) became apparent on day five postinfection, and the numbers continued to decline until the end of the experiment. On average, on day five postinfection, 81±13 percent of CD4$^+$ T cells relative to matched uninfected samples remained in LAV.04-infected tissue, and they declined further to 3±0.5 percent on day 13 postinfection. The kinetics of CD4$^+$ T cell depletion in HIV-infected tissues treated with nevirapine differed dramatically. After nevirapine was added, the numbers of infected cells gradually decreased but remained at a level of about 10 percent of CD4$^+$ T cells. However, the depletion of uninfected (p24-) CD4$^+$ T cells stopped after nevirapine was applied, and CD4$^+$ T cell numbers remained stable. On average, CD4$^+$ T cells in nevirapine-treated tissues on day eight and on day 13 postinfection remained at the same levels (46±12% and 47±10 percent, respectively), suggesting that uninfected cells were not undergoing apoptosis. Using an early apoptic marker Apo 2.7, we confirmed the lack of bystander cell death in HIV-infected tissue blocks by evaluating apoptosis among uninfected T cells (p24-).

In conclusion, our results demonstrate that the majority of CD4$^+$ T cells that are not productively infected but reside among productively infected cells in HIV-infected lymphoid tissues are not depleted. Thus, productive HIV infection is sufficient to deplete tissue of infected CD4$^+$ T cells but not sufficient to cause the death of uninfected CD4$^+$ T cells in the context of infected human lymphoid tissue. Therefore, additional factors must contribute to bystander cell death *in vivo*. General immunostimulation may be one of several factors critical for CD4$^+$ T cell depletion in HIV-infected individuals.

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Pathogenic effects of human herpesvirus 6 in human lymphoid tissue *ex vivo*

Grivel, Chen, Ito, Margolis; in collaboration with Lusso

Human herpesvirus 6 (HHV-6) is a potentially immunosuppressive agent that has been suggested to act as a cofactor in the progression of human immunodeficiency virus (HIV) disease. We have found that HHV-6 inhibits HIV-1 infection in coinfected human tissues *ex vivo*. Here, we used *ex vivo* lymphoid tissue to investigate the cellular tropism and pathogenic mechanisms of HHV-6. We sought to determine (1) whether the lymphoid tissue system can efficiently support the growth of both HHV-6 subgroups (A and B) in the absence of exogenous stimuli; (2) which cell subsets are primarily targeted by HHV-6 A and which by B; (3) whether naïve and memory T cells show a difference in susceptibility; (4) whether the infection is cytopathic for tissue-residing lymphocytes and whether it alters the expression of functionally important cell surface molecules such as the viral receptors CD46, CD3, and CD4; and (5) whether HHV-6 modulates the expression of selected chemokines. Both HHV-6 GS, a subgroup-A isolate, and HHV-6 PL1, a subgroup-B isolate, replicate in this *ex vivo* system without exogenous stimulation as evaluated with a quantitative-calibrated real-time PCR assay. As detected by flow cytometry, productive HHV-6 infection was confirmed from the intracellular expression of the viral pp41/38 antigen.

We investigated the lineage of cells productively infected by HHV-6 in lymphoid tissue by means of multicolor flow cytometry using mAbs directed against several leukocyte membrane antigens. The vast majority of viral antigen–positive cells were $CD2^+$, with very limited productive infection in other cell types. The mean proportions of productively infected cells among $CD4^+$ T cells and $CD8^+$ T cells were similar; nevertheless, given that $CD4^+$ T cells represent the predominant T cell subpopulation in human lymphoid tissue, the majority of the infected cells expressed a $CD4^+$ phenotype. Infection of $CD4^+$ T cells occurred with a similar efficiency with the two viral subgroups, whereas $CD8^+$ T cells were efficiently infected only by HHV-6 A. Analysis of the cellular phenotype in control tissues showed a prevalence of non-naïve T cells of the $CD45RA^-CD62L^+$, $CD45RA^-CD62L^-$, or $CD45RA^+CD62L^-$ phenotype, with a mean ratio of non-naïve to naïve ($CD45RA^+CD62L^+$) T cells of 2.5±0.3. Statistical analysis of the relative levels of HHV-6 infection in the two cell subsets revealed a preferential infection of non-naïve cells by both the A and B variants of HHV-6.

To evaluate the cytopathic effects of HHV-6 in human lymphoid tissue *ex vivo*, we measured the absolute numbers of cells recovered from pooled tissue fragments. HHV-6 A depleted $CD4^+$ and $CD8^+$ T cells with similar efficiency while HHV-6 B depleted $CD4^+$ T cells with a significantly greater efficiency. The data indicate that, in the context of human lymphoid tissue, HHV-6 is cytopathic and that cell depletion involves primarily the cells in which HHV-6 is replicating, without significantly depleting uninfected bystander cells. HHV-6 induces a dramatic and generalized downmodulation of its receptor, CD46, in lymphoid tissue. A marked downmodulation of CD46 expression was observed both in CD2 T cells expressing viral antigens and HHV-6-antigen–negative T cells ("bystanders"). Downmodulation of CD46 in bystander cells was further confirmed by analysis of CD46 expression in non–T ($CD2^-$) lymphoid cells. The data demonstrate that HHV-6, particularly of subgroup A, induces a marked loss of CD46 in human lymphoid tissue, which may result in an increased susceptibility to tissue damage secondary to spontaneous activation of the complement cascade. HHV-6 infection upregulates the production of RANTES, but not of other cytokines or chemokines, in lymphoid tissue, as assayed with multiplex Luminex technology. The amount of secreted RANTES correlated with the levels of HHV-6 replication. The increased secretion of RANTES in HHV-6–infected tissues may profoundly influence the physiology of the immune responses as well as, more specifically, the replication of different HIV-1 biological variants.

In conclusion, the dramatic effects that we documented in the lymphoid tissue model infected with HHV-6 of both the A and B subgroups provide compelling evidence, in a physiologically relevant system, that this agent can efficiently infect both memory and naïve T cells, causing downmodulation of physiologically relevant cell surface receptors in both infected and bystander cells and thereby, potentially, in immune dysregulation and immunosuppression.

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APPLICATIONS OF MASS SPECTROMETRY TO BIOPHYSICS, BIOCHEMISTRY, AND MEDICINE

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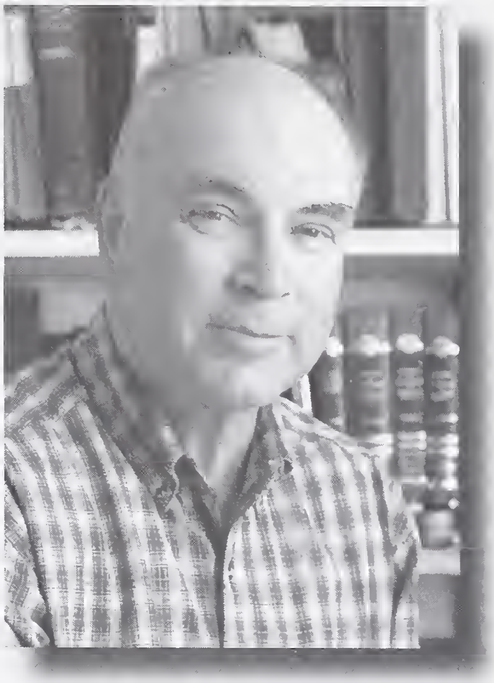

We carry out research in areas of chemistry, biochemistry, and medicine in which mass spectrometry is the primary analytic tool. Current research focuses on three areas: the reaction energetics of gas phase ions, protein characterization, and the quantification of endogenous molecules.

Reaction energetics of gas phase ions

Vieira, Gilligan, Yergey; in collaboration with Blank, Campbell, Stein, Vestal

Our work on the reaction energetics of gas phase ions has two aspects. The first is to determine the energies required for the formation of noncovalent bonds between molecules and ions in solution. The second is to understand the relationship between the energy applied in forming a peptide ion and the type and extent of its fragmentation. Knowledge of the energetics of noncovalent bonds, particularly those involving water and biologically significant molecules, is fundamental to understanding molecular interactions and their integral changes in conformations. Our research uses equilibrium ion-molecule reaction chemistry to determine the thermodynamic quantities deltaH(std), deltaS(std), and deltaG(std)298. We calculate hydration thermodynamics values from equilibrium constants measured over a temperature range of 0–136°C at water partial pressures in the ion source ranging between zero and 100 mtorr. We made equilibrium ion intensity measurements for at least four hydration states, i.e., from zero to three water molecules associated with a core ion, and include at least 60 combinations of water partial pressures and temperatures covering the range of experimental variables. We obtained results for three different classes of molecules, most recently focusing on amino acids. In the past year, we completed measurements on all 20 of the essential amino acids for clusters containing up to three water molecules each. The most striking result is that, unlike other molecular or atomic ions previously studied, the enthalpy changes associated with the successive addition of waters to any of the amino acids were virtually equivalent. Thus, the entropy changes for the successive waters are dependent on the hydration state of the amino acid ion. We interpret these results to mean that amino acid hydrations are energetically very similar but that the sites at which the water molecules are located are strongly directed by the zwitterionic nature of the ions. We have also found that the free energy for the formation of the first hydrated species correlates significantly with the published values for amino acid hydrophobicity.

In a second area of ion energetics, we have begun investigating the relationship between laser fluence and peptide ion fragmentation, a study that is fundamental to optimizing peptide sequencing by Matrix Assisted Laser Desorption Ionization Time-of-Flight with a tandem TOF mass analyzer (MALDI TOF/TOF). Accordingly, we obtain peptide fragmentation spectra, typically 5,000 laser shots, in both the unimolecular decomposition and collision-induced dissociation (CID) modes. We have the ability to follow two time points easily for each peptide of interest, i.e., the in-source fragmentation consisting of ions formed within 1 microssec after the laser firing and the longer, mass-dependent fragmentation occurring within the instrument's collision cell. To date, we have studied the fragmentation of two model peptides, leucine enkephalin (YGGFL or LeuEnk) and des-Arg-1 bradykinin (PPGFSPFR or desRB), over the full range of laser fluence available. We observe that LeuEnk exhibits an onset of fragmentation at a much lower fluence than desRB. More important, however, we have been able to demonstrate that fragment formation as a function of increasing laser fluence proceeds as a set of consecutive rather than competing reactions. The implications are particularly significant for peptide sequencing given that, in order to obtain a full set of fragment ions from a series of consecutive reactions, the peptide ion of interest must be formed with sufficient internal energy to support the entire chain of decompositions. In the context of a peptide mixture, i.e., the real situation when sequencing a peptide, the necessary amount of internal energy can be supplied only in a MALDI experiment. We are simultaneously developing a kinetic model for these decompositions by using the Rice-Ramsberger-Kassel-Marcus (RRKM) formalism for gas phase kinetics. In addition to modeling fragmentation, the calculations will define a lower limit of the peptide ion temperatures. With this information, we will be able, for the first time, to estimate the fraction of laser energy delivered to gas phase ions. Furthermore, we will have a means, other than pure empiricism, to select and optimize both MALDI matrix and laser frequency.

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Protein characterization

Backlund, Gilligan, Gordin, Vieira, Yergey; in collaboration with Blank, Coorssen, Crouch, DePamphilis, Epstein, Garland, Hinnebusch, Humphrey, Leppla, Levine, Poszgay, Robbins, Rouault, Sackett, Schneerson, Schuck, Sheeley, Spiegel, Zimmerberg

In the second area of interest, we conduct research on the mass-spectrometric characterization of proteins. We carry out our work collaboratively with groups in the NICHD as our first priority, but also independently. To identify unknown proteins, we use mass-spectrometric data to query genomic databases to determine if any of the protein sequences present in the database have expected proteolytic cleavage products with theoretical masses that match the empirically determined masses of the peptides generated from the unknown. Three mass-spectrometric approaches are available: MALDI-TOF mass analysis; liquid chromatography (LC) followed by electrospray ionization with mass analysis in an instrument capable of using fragmentation reactions to generate peptide sequences, i.e., LC-MS/MS; and, most recently, MALDI followed by tandem TOF analysis for the determination of peptide sequences from fragment ion spectra. With this combination of instrumentation, we are confident that, given enough material in a gel band to allow analysis of as little as 100 fmole, a positive identification can be made for a protein that is described in a database.

To improve protein characterization capabilities, we have begun providing sequence information on proteins that are not described in databases owing to database errors or incompleteness most frequently associated with organisms with unknown or partially characterized genomes. We call our sequence information approach "Complete *de novo* Sequencing of Peptides." It is novel compared with other widely used methods that find the so-called "sequence tag" for a peptide and determine between two and five amino acid residues from a peptide fragmentation mass spectrum. Our approach requires the determination of the amino acid sequence of the entire peptide, an approach that is limited to the use of a MALDI tandem TOF, and in that regard is a unique capability at the NIH. Making use of the recently upgraded MALDI tandem TOF instrument, our results show that our procedure yields highly reliable sequences for as many as seven peptides in the range of eight to 17 residues taken from the tryptic proteolysis of sea urchin proteins We are able to employ a subsequent collision-induced dissociation (CID) experiment in the tandem TOF and, under those circumstances, not only determined that a sequence identified in the first experiment can be verified but also that Leu residues can be distinguished from Ile. Using novel software designed and written in our section, we are undertaking detailed interpretation of individual fragmentation mass spectra of peptides required for this approach.

In two closely related areas, we are developing a methodologies for the identification of two types of posttranslational modifications: phosphorylation and disulfide links, developments that involve multiple mass spectrometry experiments and novel software written in our section. Given that, to determine the modifications effectively, it is essential to obtain virtually complete peptide coverage of the proteins by using digestion and mass spectrometry, the methodologies present a different complexity from sequence determination. Phosphorylation site identification involves the enrichment/isolation of phosphopeptides using metal affinity chromatography. Identification is followed by a combination of mass spectrometry in which we identify potential phosphopeptides by differences in ion intensities between positive and negative ion MALDI TOF spectra and then couple them with identifying amino acids residues undergoing neutral phosphate losses in using LC-MS/MS or tandem TOF approaches. We identify the sites of disulfide bridges in a somewhat similar fashion. We compare MADLI TOF spectra of proteolytic peptides obtained from reduced and unreduced proteins and then sequence peptides in the spectrum of the reduced material that are not present in the unreduced material. We then investigate potential pairing of the peptides with masses that are unique to the unreduced material.

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Ratio of endogenous cortisone to cortisol

Li, Yergey; in collaboration with Chrousos, Hochberg, Zoumakis

We use mass spectrometry to assess the activity of 11-hydroxysteroid dehydrogenase (HSD) types I and II in both cell culture and clinical studies and recently showed that HSD-I contributes to a hormonally dependent obesity when up-regulated in peripheral fatty tissue. The obesity is a consequence of the enzyme's conversion of metabolically inactive cortisone into cortisol through the reduction of a double bond in the steroid. On the other hand, HSD-II is responsible for the reverse conversion. It is hypothesized that increased activity of 11-HSD-I will be recognizable by increased levels of urinary excretion. As part of a potential therapeutic approach to treating this type of obesity, we have also undertaken an evaluation of 11-HSD-I activity in cultured fibroblasts under conditions of exposure to a variety of candidate agonists. Experimentally, we have developed an electrospray ionization LC-MS method that is capable of determining the ratio of cortisone to cortisol in both urine and culture media over a 100-fold dynamic range at picomole levels of material injected on column. Preliminary results of our studies have been satisfactory for the purposes of analyzing tissue culture media and for the determination of a suite of urinary steroids.

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MEMBRANE REMODELING DURING PATHOGENESIS AND THE MECHANISM OF EXOCYTOSIS

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The overall aim of our research is to understand membrane remodeling at the physico-chemical level. On the physical level, we generalize membrane fusion as a behavior of phospholipid membranes. On the biological level, we can divide membrane remodeling events topologically into intracellular and extracellular events. From the viewpoint of trying to quell viral infection, we study in great detail the fusion mediated by the influenza hemagglutinin (HA). From the viewpoint of understanding synaptic function, insulin release, and fertilization, we study the calcium-dependent exocytosis of the sea urchin egg.

A new viral vector for gene therapy

Kumar, Bradow[a]

Unlike oncoretroviruses, lentiviral vectors can insert large genes and can target both dividing and nondividing cells; thus, they hold unique promise as gene transfer agents. To enhance target range, the native lentiviral envelope glycoprotein is replaced (pseudotyped) with vesicular stomatitis virus G (VSVG), and the genes of interest are packaged in nonreplicating vectors by transient transfection with three plasmids. However, because of cytotoxic effects of VSVG expression in producer cells (293T cells), it has been difficult to generate a packaging cell line required for even modest scale-up of vector production. Here, we introduce a pseudotyped lentivirus vector by using the baculovirus GP64 envelope glycoprotein. Compared with VSVG, GP64 vectors exhibited a similar broad tropism and similar native titers. GP64-pseudotyped vectors were usually highly concentrated without much loss of titer. Because, unlike VSVG, GP64 expression does not kill cells, we generated 293T-based cell lines constitutively expressing GP64. Our results demonstrate that the baculovirus GP64 protein is an attractive alternative to VSVG for viral vectors used in the large-scale production of high-titer virus required for clinical and commercial applications.

Kumar M, Bradow BP, Zimmerberg J. Large-scale production of pseudotyped lentiviral vectors using baculovirus GP64. *Hum Gene Ther* 2003;14:67-77.

Molecules mediating apoptosis make lipidic pores

Basanez,[b] Chanturiya; in collaboration with Hardwick, Sharpe, Shinnar

During apoptotic cell death, cells usually release apoptogenic proteins such as cytochrome c from the mitochondrial intermembrane space. During apoptosis, Bax-type proteins cause permeabilization of the outer mitochondrial membrane to release mitochondrial apoptogenic factors into the cytosol via a poorly understood mechanism. We have proposed that Bax and Delta-N76Bcl-xL (the Bax-like cleavage fragment of Bcl-xL) function by forming pores that are at least partially composed of lipids (lipidic pore formation). We study the behavior of another peptide, the hagfish cathelicidin antimicrobial peptide, and have found that its properties are similar to those of the pro-apoptotic protein Bax.

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Formation of lipid bilayer nanotubules and their metastable transformation to catenoidal microtubules

Frolov, Lizunov; in collaboration with Dunina-Barkovskaya

We have studied shape dynamics and permeability of a membrane neck connecting a vesicle and plasma membrane. The neck is modeled by a lipid membrane tubule extended between two parallel axisymmetric rings. Within a range of lengths, defined by system geometry and the mechanical properties of the membrane, the tubule has two stable shapes, catenoidal microtubule and cylindrical nanotubule. Their permeabilities, measured as ionic conductivity of the tubule interior, differ by up to four orders of magnitude. Near the critical length, the transitions between the shapes occur within less than a millisecond. Theoretical estimates show that the shape switching is controlled by a single parameter, the tubule length. Thus, the tubule connection can operate as a conductivity microswitch toggling the release of vesicle content incellular processes such as kiss-and-run exocytosis. In support of this notion, we demonstrate bistable behavior of membrane connections between vesicles and the cell plasma membrane in macrophages.

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New method for culturing *Plasmodium falciparum*, the causitive agent of malaria

Li, Glushakova, Chanturiya, D. Yin

The traditional method of culturing *Plasmodium falciparum* has been successful. However, plasmodia replicate poorly at erythrocyte densities greater than a hematocrit of 20 percent. We have continued to develop new culture modalities, including a method to culture the major malaria parasite in a hollow fiber bioreactor that preserves healthy erythrocytes at a hematocrit up to 100 percent. In addition, these densities of parasite are allowing us to study the release of the parasite from the infected cells.

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Mechanism of influenza virus entry during infection

Frolov, Kumar, Biswas, Alfers, Babcock, Farrington, S. Yin; in collaboration with Reese

All enveloped viruses inject their genome into the cytoplasm via fusion of their envelope membrane with that of the host. Influenza virus infection, for example, follows endocytic uptake of a virus particle into a cellular vesicle that then transports the viral particle into the cell. While biological membrane fusion is classically defined as the leak-free merger of membranes and contents, both experimental and theoretical studies find that leakage occurs. The fusion stages, if any, that allow membrane permeation are uncharted. In this study, we monitored membrane ionic permeability at early stages of fusion mediated by the fusogenic protein influenza hemagglutinin (HA). HAb2 cells, expressing HA on their plasma membrane, fused with human red blood cells, cultured liver cells PLC/PRF/5, or planar phospholipid bilayer membranes. With a probability that depended on the target membrane, we generally detected an increase of the electrical conductance of the fusing membranes (leakage) by up to several nS. The leakage was recorded at the initial stages of fusion, when fusion pores formed, and usually accompanied the "flickering" stage of the early fusion pore development. As the pore widened, the leakage delined; concomitantly, the lipid exchange between the fusing membranes accelerated. We conclude that, during fusion pore formation, HA locally and temporarily increases the permeability of fusing membranes. Subsequent rearrangement in the fusion complex leads to the resealing of the leaky membranes and enlargement of the pore.

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The biochemistry and components of exocytosis

Bezrukov, Blank, Humphrey, Whalley; in collaboration with Backlund, Coorssen, Yergey

SNAREs such as VAMP, SNAP-25, and syntaxin are essential for intracellular trafficking, but we do not understand their molecular roles or their interactions with other proteins. Capitalizing on the differential sensitivities of SNAREs to exogenous proteases, we quantified the selective removal of identified SNAREs from native secretory vesicles without loss of fusion competence. Using previously established fusion assays and a high-sensitivity immunoblotting protocol, we analyzed the relationship between these SNARE proteins and Ca^{2+}-triggered membrane fusion. Neither the extent of fusion nor the number of intermembrane fusion complexes per vesicle was correlated with the measured density of identified egg cortical vesicle (CV) SNAREs. Without syntaxin, CVs remained fusion competent. Surprisingly, for one (but not another) protease, the Ca^{2+} dependence of fusion was correlated with CV SNARE density, suggesting a native protein complex that associates with SNAREs, the architecture of which ensures high Ca^{2+} sensitivity. As SNAREs may function during CV docking *in vivo* and as further proteolysis after SNARE removal eventually ablates fusion, we hypothesize that the triggered steps of regulated fusion (Ca^{2+} sensitivity and the catalysis and execution of fusion) require additional proteins that function downstream of SNAREs.

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Theoretical considerations for membrane fusion and fission

Zimmerberg; in collaboration with, Chizmadzhev, Kuzmin, Nossal

While continuing our theoretical work on membrane fusion during exocytosis, we have also carried out theoretical work on the processes of membrane fission essential to endocytosiss. The biochemical and biophysical mechanisms of membrane remodeling are critically dependent on the composition of the local piece of membrane called on by the cell to roll up into a new biological entity. The time is ripe for a detailed study of the ways that physical forces and cell membrane inhomogeneities team up to allow for controlled and organized vesiculation in the general vacuolar system of cells, the release of infectious viral particles, and the internalization of membrane-bound material. We have also proposed a new model for the cycle of exocytosis and endocytosis that can explain the regulation of transient fusion during exocytosis. With nonstructural curving proteins such as epsin, which can change the spontaneous curvature (Js) of a membrane by insertion, it is possible to imagine a scenario by which cells regulate the activity of these curving proteins to effect local budding and flattening of membranes in endocytosis and exocytosis. For example, PIP2 accumulation may lead to epsin binding, thus increasing Js and the free energy of the patch of membrane (E). The lipids respond by budding into a vesicle with the same actual curvature (J), thereby relieving the bending stress. Clathrin, which binds to epsin, could then polymerize into a cage around the vesicle, perhaps displacing epsin (which may remain at the neck to facilitate pinching off). When the vesicle was completely detached, it would uncoat. Once epsin and clathrin were gone, the spontaneous curvature of the bilayer would return to zero, but as the membrane was actually curved, stress would return and the vesicle become metastable (E=500 kBT), providing a stressed vesicle with a driving force for flattening out during exocytosis. For any vesicle about to fuse, by regulating the spontaneous curvature of the membrane through proteins such as epsin, cells could regulate the degree to which fusion pores open and remain small or repeatedly open and close ("kiss and run") rather than flattening out in complete fusion.

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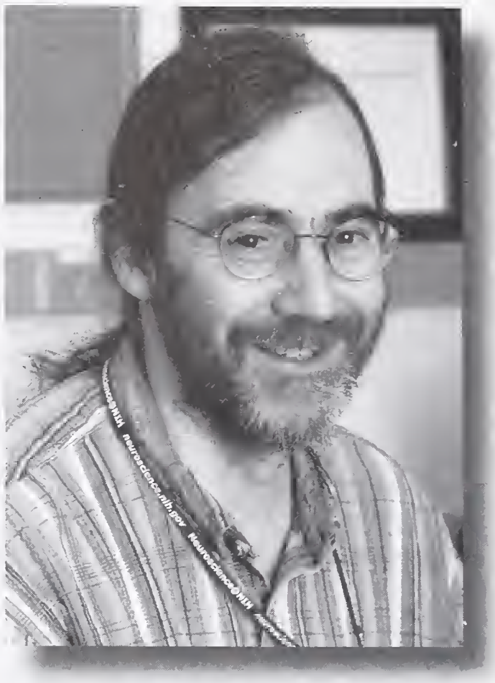
STRUCTURE AND FUNCTION OF GLUTAMATE RECEPTORS



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Ionotropic glutamate receptors (GluRs) are molecular pores that mediate signal transmission at the majority of excitatory synapses in the mammalian nervous system. Given their essential role in normal brain function and development as well as an increasing body of evidence suggesting that dysfunction of GluR activity mediates damage during stroke and multiple CNS diseases, we direct a substantial effort toward analysis of GluR function at the molecular level. The ultimate goal of our work is to obtain atomic resolution structural data, which help us define the mechanisms underlying ligand recognition and gating. Identification of the mechanisms will permit the development of subtype-selective antagonists and allosteric modulators with novel therapeutic applications.

Crystallization of kainate and delta receptor ligand binding cores

Mayer, Bessis, Molchanava

The ionotropic glutamate receptors in humans are encoded by seven gene families named after the ligands that were first used to identify the major subtypes on a functional basis: AMPA, kainate, and NMDA. The recent crystallization of the ligand binding cores of an AMPA receptor subunit and a related bacterial receptor from the photosynthetic bacterium *Synechocystis* PCC 6803 have revealed for the first time the molecular mechanisms underlying the binding of agonists and antagonists, providing insight into the mechanisms of activation and desensitization. During the past year, we have focused our experimental efforts in structural biology on similar studies on members of the kainate receptor gene family as well as on the delta receptor orphan subunits.

Ongoing work in structural biology has focused on the expression, biophysical characterization, and crystallization of the ligand binding cores of kainate subtype GluRs. Our long-term goal is to characterize members of both the high-(KA1/KA2) and low (GluR5-GluR7)-affinity kainate receptor gene families. Based on the design of the previously characterized GluR2 and GluR0 ligand binding cores, we assembled related constructs for GluR5, GluR6, KA1, and KA2 for expression in *E. coli*. Work on GluR6 is the most advanced, and we have solved structures for complexes with glutamate, 4-methylglutamate, and quisqualate. To date, we have had no success in obtaining crystals with kainate or an antagonist. The structure of the GluR6-glutamate complex was solved by MAD at NSLS beam line X9B for SeMet-labeled protein crystallized in the orthorhombic spacegroup $P2_12_12_1$. We collected a three-wavelength data set for data to 1.72 Å, from which the program SOLVE yielded an easily interpreted electron density map. The program RESOLVE built 72 percent of the structure automatically, with the remainder built by hand in O. Two molecules in the asymmetric unit were refined without NCS restraints using CNS. We solved additional structures for native glutamate complexes in hexagonal and monoclinic forms by molecular replacement using AMORE as well as for an isomorphous orthorhombic complex. The fold for GluR6 is nearly identical to that for the AMPA receptor GluR2 and reveals an identical mode of binding for glutamate, including a series of conserved water molecules in the ligand binding pocket. Further analysis is required to refine the map of subsites in the GluR6-agonist binding pocket. Given that complexes with kainate have proved difficult to crystallize, we performed soaking experiments on the orthorhombic crystal form grown in the presence of glutamate and then soaked in a glutamate-free solution containing kainate. The initial electron density maps calculated after one round of refinement revealed density in the ligand–binding site that matched that expected for glutamate, with no density corresponding to the heterocyclic ring of kainic acid, thus indicating that the orthorhombic GluR6 glutamate crystal complex is unable to exchange ligands. The result is different for GluR2, for which crystallographic experiments have demonstrated AMPA and willardiine exchange. In view of the importance of obtaining the structure of the kainate complex, our plans call for testing the hexagonal form in soaking experiments and continuing our attempts at co-crystallization with kainate as well as with antagonists.

For both GluR6 and GluR5, we completed comprehensive ligand-binding assays by using [^{3}H] kainate and [^{3}H] 4-methyglutamate for binding and displacement assays with purified S1S2 proteins. The results reveal conservation of kainate receptor subtype selectivity by the isolated GluR5 and GluR6 ligand–binding cores with, for example, binding of ATPA to GluR5 with a nM dissociation constant but no measurable binding to GluR6. Unfortunately, at present, GluR5 is proving resistant to crystallization.

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Structural basis for glutamate receptor gating

Horning, Mayer

Our functional studies over the same period had two goals: first, an analysis of the mechanism of partial agonist action and, second, identification of contacts in the dimer interface of the GluR2 AMPA receptor ligand binding core responsible for maintaining the active state of the receptor. Building on earlier work, we established that 5-substituted willardiines act as partial AMPA receptor agonists for which the extent of activation and desensitization are inversely related to the size of the 5-position substituent. Crystallographic studies revealed that, due to steric hindrance, large substituents prevent domain closure of the agonist-binding core to the same extent as that produced by full agonists. Correlated with our findings, single-channel studies revealed that full and partial agonists activate the same subconductance states, but with different relevant occupancy. Our studies on the GluR2 ligand–binding core dimer interface used the crystal structure as a basis for designing mutants intended to test the role of ion pair interactions, hydrogen bonds, and van der Waals contacts of hydrophobic side chains in maintaining the active state. The results are in excellent agreement with those predicted by the crystal structure and reinforce our earlier proposal that the dimer interface is a critical structural element in the gating mechanism.

Previous work combining X-ray diffraction, ultracentrifugation, and patch-clamp physiology has shown that the strength of dimer formation by GluR2 ligand binding cores controls desensitization. Within the dimer interface, there are extensive contacts between the adjacent ligand-binding cores, including a pair of salt bridges between E486 and K493, as well as hydrogen bond and van der Waals contacts between helices D and J. To determine the residues involved in the reorganization of dimer interface contacts that leads from the active state to the desensitized state, we used alanine-scanning and site-directed mutagenesis of the GluR2 dimer interface in combination with patch-clamp analysis. Truncation of the lysine at site 493 to an alanine (K493A) generated a 41-fold faster rate of desensitization compared with the wild-type receptor while the rate of recovery from desensitization decreased by a factor of three. At the site (K493M) we introduced a methionine residue that possesses similar shape and volume to lysine yet lacks the positive charge; the K493M mutant increased the rate of desensitization by a factor of 11 compared with wild type, indicating that both the size and charge of the lysine side chain play a role in stabilizing the dimer interface. To shorten the side chain and eliminate the negative charge of the counter ion in the salt bridge, we substituted glutamate residue 486 for alanine (E486A). Similar to K493M, the rate of onset of desensitization accelerated more than 10-fold while, for the glutamine and aspartate substitutions, the rates increased four-fold and 46-fold, respectively. The modest change observed for E486Q indicates that hydrogen bond formation with K493 substitutes for loss of the ion pair interaction. Conversely, when the distance between the carboxyl and butylamino side chains is increased in the E486D mutant, the interface is strongly perturbed. Less disruption occurs in the K493M, E486A, and E486N mutants, indicating that it is energetically unfavorable to bury the isolated charge of the delta carboxyl side chain in the E486D mutant.

A conserved hydrophobic cluster is found in the GluR2 dimer that is formed by residue L483 in helix D in one protomer and by residues L748, L751, and K752 projecting from helix J of the adjacent protomer. Mutation of site 748 to an alanine (L748A) resulted in a 13-fold higher rate of onset of desensitization compared with wild type while, for L751A, responses could not be obtained except in the presence of the allosteric modulator cyclothiazide, which binds at the base of the dimer interface. For K752A and L482A, the rate of desensitization was four- to five-fold slower than wild type. This surprising result can perhaps be explained by the removal of steric constraints, allowing closer apposition of the pair of protomers and increasing the extent or strength of van der Waals contacts.

A complex network of hydrogen bond contacts also contributes to GluR2 dimer formation. The side chain of E486 interacts with helix J via the side chain of N747; elimination of this hydrogen bond in the N747A mutant increases the rate of onset of desensitization nearly 20-fold. Surprisingly, for the N747D mutant, the rate of desensitization increased only nine-fold, indicating that coulombic repulsion did not occur between residues E486 and N747D. At the base of helix J, the side chain of E755 makes direct and solvent-mediated hydrogen bonds with the main-chain amide nitrogens of L483 and V474, respectively. Partial disruption of hydrogen bond contacts in the E755Q mutant accelerated the rate of onset of desensitization 20-fold while the E755D, E755N, and E755A mutants responded only in the presence of the allosteric modulator cyclothiazide. In combination, our results greatly strengthen the evidence for earlier proposals that dimer formation by the GluR2 ligand binding core is a critical structural element in the gluR2 gating process. Our studies go beyond the earlier findings and identify for the first time some of the major contacts in the dimer interface.

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POTASSIUM CHANNELS AND DENDRITIC FUNCTION IN HIPPOCAMPAL PYRAMIDAL NEURONS

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A nonuniform density of subthreshold, rapidly inactivating potassium channels regulates signal propagation in the dendrites of CA1 pyramidal neurons of the hippocampus. This nonuniform distribution (with higher expression in the dendrites than in the soma) means that the electrical properties of the dendrites differ markedly from those of the soma. Incoming synaptic signals are shaped by the activity of these channels, and action potentials, once initiated in the axon, progressively decrease in amplitude as they propagate back into the dendrites. By combining patch-clamp recording in brain slices of the hippocampus with molecular biology techniques, we investigate the electrophysiological properties and molecular nature of the voltage-gated channels expressed in CA1 dendrites, how their expression is regulated, and their role in synaptic integration and plasticity.

Creation and characterization of a Kv4.2 transgenic mouse

Hoffman, Kim

We are currently characterizing a transgenic mouse expressing a dominant-negative pore mutation in the voltage-gated potassium channel subunit Kv4.2. The mouse expresses the mutant channel along with GFP under control of a tetracycline transactivator (tTA)–responsive promoter. A new line of tTA-expressing mice that limit tTA activity to the CA1 and dentate gyrus regions of the hippocampus controls expression spatially. Expression can also be controlled temporally by administration of doxycycline.

Kv4.2 trafficking in CA1 pyramidal neuron dendrites

Kim, Hoffman

Although the molecular identity of the nonuniform density of subthreshold, rapidly inactivating potassium channels found in hippocampal CA1 pyramidal neuron dendrites is not known for certain, antibody and *in situ* data implicate Kv4.2. Using GFP-fused Kv4.2 constructs, we are studying Kv4.2 trafficking to the dendrites.

Role of voltage-gated potassium channels in synaptic plasticity

Beck, Kim, Hoffman

Potassium channels have been shown to regulate the back-propagation of action potentials (BPAps) into CA1 dendrites. Although the functional role of BPAps is unclear at this time, it has recently been suggested that they may provide the depolarization necessary to unblock NMDA receptors, allowing for the induction of synaptic plasticity. We are currently investigating the effect of potassium channel mutations on BPAps and in the induction of LTP in organotypic slice cultures from wild-type and transgenic mice.

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NEUROTROPHIC REGULATION OF SYNAPSE DEVELOPMENT AND PLASTICITY

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We study the molecular mechanisms underlying synapse development and function, with particular emphasis on the role of neurotrophic factors. Traditionally, neurotrophic factors have been defined as secretory proteins that regulate neuronal survival and differentiation. Recent studies indicate that a major function of neurotrophic factors in the brain is to regulate synaptic transmission and plasticity. Our laboratory was among the first to reveal this novel function. Using rodent models, we demonstrated that brain-derived neurotrophic factor (BDNF) plays a pivotal role in hippocampal long-term potentiation (LTP), a cellular model for learning and memory. Using the *Xenopus* neuromuscular synapse as a model, we identified two modes of regulation by neurotrophins: *acute* modulation of synaptic transmission and plasticity and *long-term* alteration of the structure and function of synapses. In the past year, we have focused on a major cell-biological issue: how diffusible molecules such as neurotrophins elicit local and synapse-specific modulation. We have also investigated the signaling mechanisms mediating the regulatory effects of neurotrophic factors on synapses. In addition, we have made several unexpected discoveries about the mechanisms for synaptic transmission and synapse development.

Cell biological mechanisms ensuring synapse-specific modulation by BDNF

Zaistev, Du,[a] *Je, Lu; in collaboration with Egan, Kojima, Weinberger*

Previous studies by our and other laboratories indicate that BDNF is important for hippocampal synaptic plasticity and hippocampus-dependent learning and memory in animals. To examine the relevance of these studies in the human, we studied a human single nucleotide polymorphism (SNP) that converts a valine (val) to methionine (met) in the 5′ pro-region of the BDNF protein. Subjects with the met/met genotype exhibited poorer performance in tests of episodic memory. In subjects with a met allele, functional magnetic resonance imaging (fMRI) of two separate cohorts both revealed abnormal hippocampal activation during a cognitive task. Furthermore, levels of n-acetyl aspartate (NAA), a putative *in vivo* measure of neuronal integrity and synaptic abundance, were lower in the hippocampal formation of subjects with met alleles than in that of normal subjects. Rodent hippocampal neurons transfected with val-BDNF-GFP exhibited a punctate distribution pattern throughout the soma and dendrites while those transfected with met-BDNF-GFP showed large clusters in the perinuclear region. Double staining experiments indicated that met-BDNF-GFP failed to localize in secretory granules or to be transported to the synapses. Depolarization-induced secretion of met-BDNF decreased significantly while its constitutive secretion remained unchanged. The results represent the first demonstration of a role for BDNF in human memory and hippocampal function and suggest that val/met exerts these effects by influencing synaptic targeting and activity-dependent BDNF secretion. Thus, it is the local and activity-dependent secretion of BDNF at synapses, rather than the level or secretion of BDNF per se, that is critical for hippocampal plasticity and memory function.

Another mechanism for achieving synapse-specific modulation is for activity-dependent control of neurotrophin signaling to occur through membrane receptors. We have previously shown that high-frequency neuronal activity facilitates the insertion of the BDNF receptor TrkB into the cell surface, leading to an enhanced response to BDNF in active neurons/synapses. We have now examined the role of neuronal activity in TrkB internalization, a process critical for many aspects of BDNF functions. Using three independent approaches, we showed that electrical stimulation of hippocampal neurons markedly enhances TrkB internalization. Electrical stimulation also potentiates TrkB tyrosine kinase activity. The activity-dependent enhancement of TrkB internalization and its tyrosine kinase requires Ca^{2+} influx through NMDA receptors and Ca^{2+} channels. Inhibition of internalization has no effect on TrkB kinase, but inhibition of TrkB kinase prevents the modulation of TrkB internalization, suggesting a critical role of the tyrosine kinase in the activity-dependent receptor endocytosis. Assuming that the mechanism is operative at synapses, BDNF may preferentially modulate active synapses by activity- and Ca^{2+}-dependent potentiation of TrkB tyrosine kinase and its internalization.

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Signaling mechanisms underlying synaptic modulation by neurotrophic factors

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Clustering of neurotransmitter receptors in the postsynaptic membrane is critical for synapse formation and maintenance. The key neurotrophic factor agrin induces the clustering of acetylcholine receptor (AChR) at the neuromuscular junction (NMJ). The effect of agrin is mediated by the transmembrane tyrosine kinase MuSK. However, the signaling mechanism underlying MuSK is poorly characterized. We show that geranylgeranyltransferase I (GGT) is an important signaling component in the agrin/MuSK pathway. Agrin stimulation of MuSK causes a rapid increase in tyrosine phosphorylation and activity of GGT, leading to prenylation and therefore membrane translocation of Rho, a small GTPase required for AChR clustering. Inhibition of GGT function or expression inhibits agrin-induced activation of Rho, prevents muscle cells from forming AChR clusters in response to agrin, and attenuates the formation of neuromuscular synapses in motoneuron-muscle co-cultures. Importantly, transgenic mice expressing the dominant negative GGT K164A demonstrate NMJ defects with wider end-plate bands and smaller AChR plaques. The results support the notion that prenylation is necessary for AChR clustering and NMJ formation and/or maintenance and reveal an active role of GGT in agrin/MuSK signaling.

Neuregulin is another neurotrophic factor known to control the synaptic synthesis of AChR at the NMJ. Recent studies indicate that neuregulin also regulates hippocampal synaptic plasticity. It is unclear how neuregulin signaling is targeted to synapses. We show that the neuregulin receptor ErbB4, which is localized to postsynaptic densities (PSD) in the brain, is recruited to the neuronal lipid raft fraction upon stimulation by neuregulin. Along with ErbB4, its adapter proteins Grb2 and Shc are translocated to lipid rafts by neuregulin stimulation. The partitioning of ErbB4 into lipid rafts is enhanced through interaction between the ErbB4 C-terminus and the PDZ domains of PSD-95, a molecule residing in PSD. Disruption of lipid rafts inhibits neuregulin signaling through MAP kinase and prevents neuregulin regulation of hippocampal LTP. Thus, ligand-induced translocation of ErbB4 into lipid rafts appears to be an important step in neuregulin signaling and regulation of hippocampal synapses.

TGF-betas are neurotrophic factors that play an important role in brain development. Smad4 is a central mediator of all TGF-betas. Using a Cre-loxP approach to overcome early embryonic lethality, we have studied functions of TGF-beta/Smad4 signals in the central nervous system (CNS). We detected no obvious deficits in mice carrying the targeted disruption of *Smad4* in the CNS. The overall morphology of the hippocampus appeared normal. We observed no change either in the proliferation of neuronal precursor cells or in several forms of synaptic plasticity. In contrast, deletion of *Smad4* results in a marked decrease in the number of cerebellar Purkinje cells and parvalbumin-positive interneurons. Accompanied by the abnormality in the cerebellum, mutant mice also exhibit significantly increased vertical activity. Thus, our study reveals an unexpected role of Smad4 in both cerebellar development and the control of motor function.

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Unexpected mechanisms in controlling transmitter release and synaptic development

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In addition to studying neurotrophic regulation, we are interested in the basic mechanisms underlying the development and function of synapses. One of the essential mechanisms is the activity-dependent modulation of synaptic transmission. We have recently identified an unusual form of synaptic modulation that depends on Na^+ influx and a mitochondrial Na^+-Ca^{2+} exchanger, but not on Ca^{2+} influx. In Ca^{2+}-free medium, tetanic stimulation of *Xenopus* motoneurons induced a striking potentiation of transmitter release at neuromuscular synapses. Inhibition of either Na^+ influx or the rise in Ca^{2+} concentrations ($[Ca^{2+}]i$) at nerve terminals prevented tetanus-induced synaptic potentiation (TISP). Blockade of Ca^{2+} release from the mitochondrial Na^+-Ca^{2+} exchanger, but not from endoplasmic reticulum Ca^{2+} stores, also inhibited TISP. Tetanic stimulation in a Ca^{2+}-free medium elicited an increase in $[Ca^{2+}]_i$, which was prevented by inhibition of Na^+ influx or mitochondrial Ca^{2+} release. Inhibition of protein kinase C (PKC) blocked tetanus-induced synaptic potentiation as well as mitochondrial Ca^{2+} release. The results reveal a novel form of synaptic plasticity and suggest a role of PKC in mitochondrial Ca^{2+} release during synaptic transmission.

We have also studied the basic mechanisms governing synapses development. Specifically, we have examined the role of the cell adhesion molecule beta-catenin in the formation of hippocampal synapses. At presynaptic terminals, synaptic vesicles can be divided into two pools: a reserved pool (undocked vesicles) and a releasable pool (vesicles docked at presynaptic membrane). Deletion of beta-catenin in hippocampal pyramidal neurons *in vivo* resulted in a lower number of undocked synaptic vesicles per synapse and an impaired response to prolonged-repetitive stimulation. The result corresponded to the diffuse expression of vesicles along the axon *in vitro*. Interestingly, the effects are not due to beta-catenin's involvement in N-cadherin–mediated adhesion or Wnt signaling. Instead, beta-catenin modulates vesicle localization by acting as a scaffold to link PDZ proteins with cadherin via its PDZ binding domain. The study defines a specific role for cadherins and catenins in synapse organization beyond their role in mediating cell adhesion.

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HIPPOCAMPAL INTERNEURONS AND THEIR ROLE IN CONTROLLING EXCITABILITY

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GABAergic inhibitory interneurons constitute a population of hippocampal cells whose high degree of anatomical and functional divergence make them suitable candidates for controlling the activity of large populations of principal neurons. GABAergic inhibitory interneurons play a major role in the synchronization of neuronal activity and are involved in the generation of large-scale network oscillations. Thus, interneurons function as a clock that dictates when principal cells fire during suprathreshold excitatory drive. Interneurons receive strong excitatory glutamatergic innervation via numerous anatomically distinct afferent projections, and recent evidence has demonstrated that the molecular composition of both classes of AMPA-preferring glutamate receptors expressed at interneuron synapses are often distinct from those found at principal cell synapses. Furthermore, single inhibitory interneurons can synthesize distinct AMPA receptors with defined subunit composition and target them to synaptic domains innervated by different afferent inputs. Using high-resolution whole-cell patch-clamp recording techniques in brain slices of hippocampus and auditory and barrel cortex, we investigate differential mechanisms of synaptic transmission onto hippocampal inhibitory interneurons and the role of intrinsic voltage-gated channels in regulating interneuron excitability.

Multiple muscarine-sensitive conductances prime hippocampal CA1 st. oriens-alveus (OA) interneurons for local circuit oscillations

Lawrence, Statland

Cholinergic input induces network oscillations in the hippocampus, which is important in learning, memory, and neurodegenerative diseases. While considerable effort has gone into understanding how muscarine regulates pyramidal cells, muscarine's effects on interneurons are less well understood. In this study, we investigated the effects of muscarinic receptor activation on action potential generation in CA1 OA interneurons and the ionic conductances underlying this modulation. In an initial set of experiments, muscarine increased the firing frequency to 20-100 pA current injections (1 sec duration) in half the cells tested. The same subset of cells was also associated with an initial depolarization by muscarine, shortened first spike latency, and a relative loss of spike frequency accommodation. This excitatory effect of muscarine was lost in M2 knock-out mice (0 of 23 cells). We next examined how muscarine influences resonance properties of cells in response to oscillatory input by introducing sinusoidal currents at frequencies 0.5 to 100 Hz into the cells at near-threshold potentials. Muscarine increased spike reliability (spike probability per cycle) between 5 and 30 Hz, especially in the theta range. We then determined which ionic conductances were modulated by muscarine and found that muscarine significantly reduced the M-current (I_M), the slow afterhyperpolarization (sI_{AHP}) current, and the leak current ($I_{K(Leak)}$). We propose that, in OA interneurons, activation of the septal cholinergic system causes a shift in the balance between the I_M, sI_{AHP}, and $I_{K(Leak)}$. If the changes in I_M and sI_{AHP} predominate, the net effect is to engage the hippocampal network by increasing AP reliability to synaptic inputs. However, domination by $I_{K(Leak)}$ reduces AP reliability, promoting disengagement from the network. Thus, the same neurotransmitter may differentially affect subsets of OA interneurons to enable a theta-permissive state.

Fisahn A, Yamada M, Duttaroy A, Gan J-W, Deng C-X, McBain CJ, Wess J. Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents. *Neuron* 2002;33:615-624.

Depolarization-induced long-term depression at hippocampal mossy fiber-CA3 pyramidal neuron synapses

Lei; in collaboration with Congar, Lacaille, Topolnik

Hippocampal CA3 pyramidal neurons receive two types of excitatory afferent innervation: mossy fibers (MF) from granule cells of the dentate gyrus and recurrent collateral fibers (CF) from other CA3 pyramidal neurons. At CF-CA3 pyramidal neuron synapses, membrane depolarization paired with low (0.33 Hz) presynaptic stimulation generated a heterogeneous response that ranged from long-term potentiation (LTP), long-term depression (LTD), to no alteration of synaptic strength. However, the same induction paradigm applied at MF-CA3 pyramidal neuron synapses consistently induced LTD. This novel form of LTD was independent of NMDA receptors (NMDARs), metabotropic glutamine receptors (mGluRs), cannabinoid receptors, or coincident synaptic activity but was dependent on postsynaptic Ca^{2+} elevation through L-type Ca^{2+} channels. Ca^{2+} imaging of both proximal and distal CA3 pyramidal neuron dendrites demonstrated that the depolarizing induction paradigm differentially elevated intracellular Ca^{2+} levels. We observed L-type Ca^{2+} channel activation only at the most proximal locations where mossy fibers make synapses. Depolarization-induced LTD did not occlude the conventional 1 Hz–induced LTD or vice versa, suggesting that independent mechanisms underlie each form of plasticity. The paired-pulse ratio and coefficient of variation of synaptic transmission remained unchanged after LTD induction, suggesting that the expression locus of LTD is postsynaptic. Moreover, peak-scaled nonstationary variance analysis indicated that depolarization-induced LTD correlated with a reduction in postsynaptic AMPA receptor numbers without a change in AMPA receptor conductance. Our results imply that this novel form of LTD is selectively expressed at proximal dendritic locations closely associated with L-type Ca^{2+} channels.

Two loci of expression for long-term depression at hippocampal mossy fiber-interneuron synapses

Lei

One of our long-term goals has been to identify novel mechanisms of long-term plasticity at hippocampal interneuron synapses. Two distinct forms of LTD exist at synapses between dentate gyrus granule cells and CA3 interneurons. At Ca^{2+}-impermeable AMPA receptor (CI-AMPAR) synapses, LTD induction is NMDA receptor–dependent, whereas LTD induction at Ca^{2+}-permeable AMPA receptor (CP-AMPAR) synapses is NMDA receptor–independent. The LTD expression locus of either form, however, has not been previously examined. Using numerous criteria, we have examined the locus of expression for each form of LTD. Techniques include a comparison of the coefficient of variation and paired-pulse ratio before and after LTD expression and a comparison of AMPA/NMDA receptor activity. More recently, we took advantage of the low-affinity AMPA receptor antagonist gamma-DGG, which can be used to probe for changes in synaptic cleft glutamate following plasticity expression. Using these techniques, we demonstrated that LTD expression at CP-AMPAR synapses is presynaptic and results from a reduction in neurotransmitter release, whereas LTD expression at CI-AMPAR synapses is entirely postsynaptic. In a related series of experiments, inclusion of the NSF/AP2-inhibitory peptide pep2m in the whole-cell recording pipette blocked LTD expression at CI-AMPAR synapses, but not at CP-AMPAR synapses, confirming that CI-AMPAR LTD involves postsynaptic AMPA receptor trafficking. Thus, mossy fiber innervation of CA3 st. lucidum interneurons occurs via two parallel systems, each with a distinct expression locus for long-term synaptic plasticity.

The role of kainate receptor subunits GluR5 and GluR6 in hippocampal gamma oscillations

Fisahn; in collaboration with Buhl, Heinneman

Activation of kainate receptors (but not AMPA receptors) induces gamma oscillations in the hippocampus *in vitro*. These kainate-induced gamma oscillations depend on intact inhibitory GABAergic neurotransmission but are independent of excitatory transmission via NMDA receptors, metabotropic glutamate receptors, and cholinergic receptors. We investigated the connection between gamma oscillations and the kainate receptor subtypes GluR5 and GluR6. Using extracellular field recordings, we demonstrated that gamma oscillations of comparable power were induced by much lower concentrations of kainate in GluR5$^{-/-}$ (100nM) CA3 hippocampus than in wild type (600nM). Increasing concentrations of kainate (>150nM) degraded the gamma oscillation and precipitated electrographic seizure activity in the GluR5$^{-/-}$ hippocampus but not in wild type. In contrast, kainate failed to induce gamma oscillations or seizures at concentrations up to 600nM in the GluR6$^{-/-}$ hippocampus. Given that gamma oscillations were readily evoked by other oscillogenic drugs such as muscarine (20 micromolar), the lack of effect of kainate was not explained by altered neuronal circuitry in GluR6$^{-/-}$. We concluded that activation of the GluR6 kainate receptor subtype is necessary for the induction of gamma oscillations. Next, using whole-cell recordings, we demonstrated that kainate increased sIPSC amplitude by about 50 percent in wild type and GluR5$^{-/-}$ but was without effect on sIPSCs in GluR6$^{-/-}$. Furthermore, kainate activated an inward current and depolarized pyramidal neurons in both WT and GluR5$^{-/-}$, but not in GluR6$^{-/-}$. These effects of kainate on the hippocampal network are consistent with the expression patterns of GluR5 and GluR6, which are primarily localized to inhibitory interneuron presynaptic terminals and the somata and dendrites of interneurons and pyramidal cells, respectively. Consistent with a presynaptic expression pattern of GluR5, the paired-pulse ratio of evoked IPSCs increased, suggesting that GluR5 may act to depolarize presynaptic inhibitory terminals tonically and thus provide an elevated basal state of inhibition within the hippocampal network. Similarly, the increased sensitivity to kainate-induced oscillations in GluR5$^{-/-}$ likely results from a reduction of the inhibitory control over the CA3 network, producing an enhanced excitability of pyramidal neurons and a reduced ability of the interneuron network to synchronize itself at gamma frequencies.

Fisahn A, Yamada M, Duttaroy A, Gan J-W, Deng C-X, McBain CJ, Wess J. Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents. *Neuron* 2002;33:615-624.

Metabotropic glutamate receptor-7b regulation of mossy fiber-interneuron transmission

Pelkey

Anatomical evidence indicates that mGluR7b receptors are selectively localized to the presynaptic grid of mossy fiber terminals opposing interneurons of the CA3 hippocampus but are completely absent from the same terminals made onto CA3 principal neurons. This anatomical arrangement suggests that mGluR7 receptors provide selective control of transmission through the inhibitory arm of the mossy fiber-CA3 system. In hippocampal slices, activation of mGluR7 irreversibly depressed excitatory postsynaptic currents (EPSCs) onto st. lucidum interneurons evoked by MF stimulation. Analysis of EPSC evoked by paired pulses indicated a presynaptic effect, suggesting that mgluR7 activation depresses transmitter release rather than altering postsynaptic receptor sensitivity. We have previously shown that high-frequency stimulation paradigms that typically result in long-term potentiation of mossy fiber-pyramidal neuron synaptic transmission induce a long-term depression of mossy fiber synaptic activity onto interneurons. These activity-dependent forms of synaptic plasticity are relevant to both physiological and pathological processes within the CNS; consequently, there is much interest in uncovering cellular and molecular mechanisms governing these changes. We have now found that blockade of mGluR7 activation prevents high frequency–induced depression of mossy fiber inputs to st. lucidum interneurons, demonstrating that mGluR7 activation is both sufficient and necessary for mossy fiber-interneuron LTD induction. The data highlight a previously unexplored regulation of mossy fiber transmission by the mGluR7b receptor isoform.

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OLFACTORY CODING AND DECODING BY ENSEMBLES OF NEURONS

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All animals need to know what is going on in the world around them; thus, brain mechanisms have evolved to gather and organize sensory information and to build transient and sometimes enduring internal representations of animals' surroundings. Using relatively simple animals and focusing primarily on olfaction, our unit combines electrophysiological, anatomical, behavioral, and other techniques to examine the ways intact neural circuits, driven by sensory stimuli, process information.

In the past year, our new research program has begun to address several questions, among them: the nature of the mechanisms, including the transient oscillatory synchronization of ensembles of neurons, that underlie information coding and decoding; the nature of the neural mechanisms that underlie the phenomenon of stimulus invariance (in which stimuli are perceived as the same, or of the same class, despite actual differences in orientation, intensity, and so forth); the manner in which multimodal stimuli are integrated into unified perceptions; and how innate sensory preferences are determined. Our work reveals basic mechanisms by which sensory information is transformed, stabilized, and compared as it makes its way through the nervous system.

Stopfer M, Jayaraman V, Laurent G. Intensity versus identity coding in an olfactory system. *Neuron* 2003;39: 1-20.

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CLINICAL GENOMICS

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It is increasingly obvious that biological processes in living organisms are tightly regulated by networks of genes. The traditional one gene–one pathway is not appropriate in the study of complicated biological processes such as development. Our research uses functional genomic approaches to study germ cell and sexual development in normal and pathological states in order to understand the intricate regulatory mechanism of cellular proliferation and differentiation. Similarly, many seemingly nonhereditary diseases have now been shown to have a genetic component. In line with this observation, we have identified a genetic risk factor for pseudotumor cerebri in the Turkish population, in which heritable factors had not been previously identified.

Functional genomic studies of germ cell development in spermatogenesis

Wu, Baxendale, Pang, Johnson, Leung, Ruszczyk, Rennert, Chan; in collaboration with Dym, Ravindranath, Stitely

Spermatogenesis is a tightly regulated process characterized by spermatogonial stem cells undergoing mitotic expansion, meiosis, and postmeiotic differentiation. The distinct morphological and biological characteristics of germ cells observed at different stages of spermatogenesis allow preparation of these cells in relatively pure form. In addition, animal models permit the study of arrest and reinitiation of spermatogonial differentiation, making spermatogenesis a unique model for studying stem cells and the genetic factors that regulate cellular proliferation and differentiation in general.

By sequencing the respective SAGE (Serial Analysis of Gene Expression) libraries, we determined the transcriptome of mouse type A spermatogonia (Spga), pachytene spermatocytes (Spcy), and round spermatids (Sptd) purified by the STAPUT technique. We analyzed 444,015 SAGE tags derived from one Spga, two Spcy, and one Sptd library and identified 34,619 different species of transcripts among these SAGE tags. Among the transcripts, 42.6 percent were derived from known genes, 42.1 percent were uncharacterized cDNAs, and 15.3 percent had not been previously described. Analysis of the spermatocyte transcriptome indicates that the germ cell transcriptome could consist of more than 30,000 transcripts, different from what has been observed for other cells (Zhang *et al.*, *Science* 1997;276:1268). Some 8 to 14 percent of the transcriptome consists of novel transcripts, 44 to 46 percent consists of uncharacterized cDNAs, and 42 to 46 percent consists of transcripts of known genes. Figure 9.1 shows the distribution of transcripts among the three cell types analyzed. Some 12 to 19.5 percent of the transcriptomes are of cell-specific transcripts while approximately

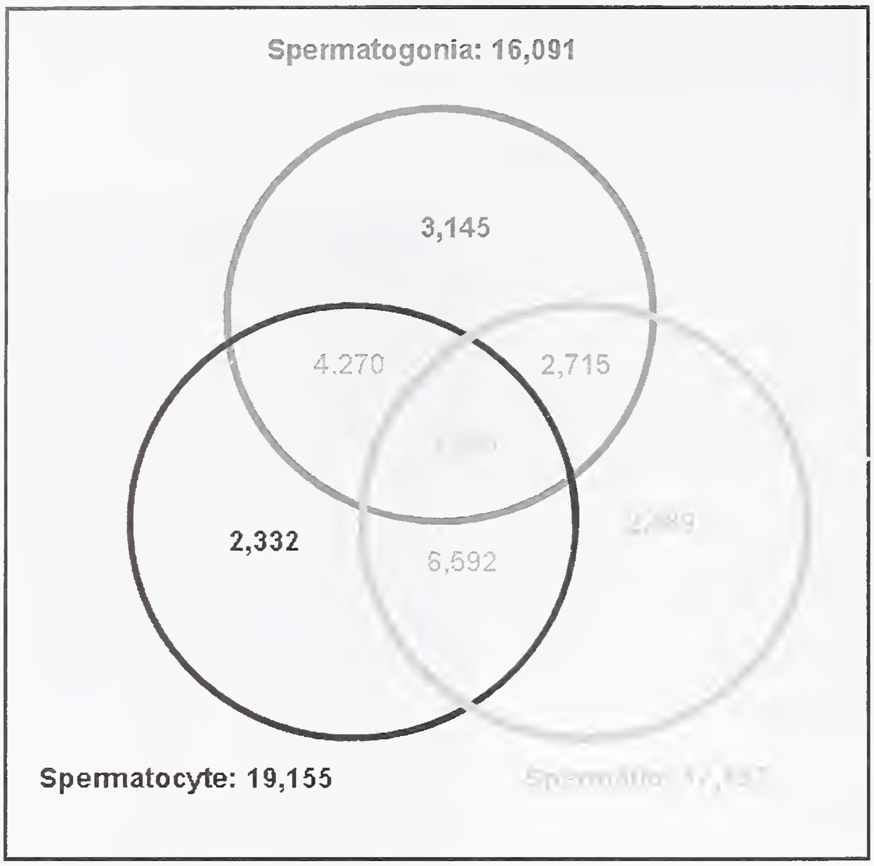

FIGURE 9.1

Overlap of the transcriptome of type A spermatogonia, pachytene spermatocytes, and round spermatids

The number of unique tags in each of the transcriptomes is indicated by the side of the name of the type of cells. The numbers in the Venn diagram represent the number of tags in the respective segments.

6,000 transcripts are shared by the three types of cells. It is worth noting that the most abundant tag matched with three UniGene clusters while the second most abundant tag represented an uncharacterized cDNA and the third most abundant tag had no match in the SAGE-map database. We validated differential expression between the SAGE libraries by quantitative real-time polymerase chain reaction (QPCR) of randomly selected transcripts. Components of the protein biosynthetic machinery are highly expressed in Spga. In Spcy, transcription factors are abundantly expressed, with chromosome-remodeling genes and testis-specific genes prominent in Sptd. We used comparative analysis of SAGE and QPCR data to reveal the presence of alternatively spliced variants. The present study is the first global study of gene expression in mouse germ cells and demonstrates that a core set of genes may be required for basic biological activities in all germ cells. However, cell-specific stages of spermatogenesis (mitotic, meiotic, and postmeiotic) require the concerted action of distinct sets of genes.

Pang A LY, Taylor HC, Johnson W, Alexander S, Chen Y, Su YA, Li X, Ravindranath N, Dym M, Rennert OM, Chan WY. Identification of differentially expressed genes in mouse spermatogenesis. *J Andrology* 2003;24:59-71.

Molecular cloning and characterization of genes in spermatogenesis

Pang, Johnson, Ruszczyk, Baxendale, Rennert, Chan; in collaboration with Dym, Ravindranath, Su

One major drawback of studies of gene expression during spermatogenesis is the lack of information on the expression of "housekeeping" genes in somatic tissues. Several reports indicate that the expression levels of some such genes would be altered under different physiological conditions, e.g., during cellular differentiation or when cells become malignant. We used QPCR analysis to examine the expression levels of 20 genes considered to be housekeeping genes in various studies of type A spermatogonia, pachytene spermatocytes, and round spermatids. Interestingly, expression levels of housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase, beta-tubulin 5, and cyclophilin D varied significantly among the three types of germ cells, whereas histone deacetylase 2 and 18S rRNA showed the least fluctuation in expression levels. This is the first study of housekeeping genes in germ cells; the data could serve as a standard for gene expression studies in distinct types of germ cells when precise gene expression measurements among these cells is needed.

It has been reported that aberrant expression of some X-linked genes would cause failure in spermatogenesis as well as improper sexual development. A recent report indicated that spermatogonia express a substantial number of X-linked genes related to spermatogenesis. We have identified several X-linked genes that escape transcription inactivation at meiosis and in the postmeiotic stage or are expressed immediately before the pachytene stage. To gain a better understanding of the regulation of X-linked gene expression, we clustered the expression of all X-linked genes identified in germ cells. Two such genes, the *Testis expressed* gene *13 (Tex13)* and *Sex comb on midleg-like 2 (Scml2)*, were expressed predominantly in type A spermatogonia. *In silico* studies indicated the existence of a potential antisense transcript of *Tex13*, which is complementary to the 3′ end of the *Tex13* sense transcript. Reverse transcription-polymerase chain reaction (RT-PCR) studies revealed that the "trend of change" in expression of the sense and antisense transcripts was similar, but relative expression levels of the transcripts were dissimilar in germ cells at different stages. Regulation of the expression, biological activities, and relationship between the sense and antisense transcripts of *Tex13* are currently under study. *Scml2* is a member of the Polycomb group of genes, which encode transcriptional repressors essential for appropriate development in the fly and in mammals. We found at least three different *Scml2* transcripts in mouse testes, each with a different 5′ untranslated region, and observed alternative use of exons in the coding region. Using RT-PCR analyses, we found that the various forms of *Scml2* transcripts were expressed at different levels during spermatogenesis, implying preferential use of the transcripts during germ cell development. *In vivo* as well as *in vitro* gene knock-down experiments are planned to study the functional roles of these X-linked genes during spermatogenesis.

Using SAGE and cDNA microarray experiments, we cloned and characterized several novel as well as uncharacterized cDNAs identified as differentially expressed in germ cells. In addition, using a cDNA library screening and a 5′ RACE, we cloned a novel transcript of about 1.1kb, shown to be preferentially expressed in round spermatids; we also cloned another spermatid-specific expressed sequence tag (EST) identified by cDNA microarray experiment. SAGE analyses of germ cell transcriptomes identified a number of novel transcripts, the more abundant of which are being cloned by RT-PCR with extended primers. Characterization and developmental studies of these cDNAs are expected to yield information on their roles in spermatogenesis.

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Functional genomic studies of gonad and early germ cell development

Vong, Baxendale, Pang, Ruszczyk, Rennert, Chan; in collaboration with Culty, Lau

Sexual dimorphism of the mouse gonad begins with expression of the *Sry* gene at around E11 (fetal day 11). When the fetal gonad is developing, primordial germ cells are moving and by E13 arrive at the genital ridge. Differentiation of the male and the female gonads and the concomitant development of the gonocytes and oocytes involve the intricate interaction of a large number of genes; the identity of the majority of these genes is still largely unknown. We have undertaken functional genomic studies using both SAGE and oligonucleotide microarray hybridization to delineate this phenomenon. Sexing of early gonads was done by RT-PCR to detect the expression of *Sry* while that of later gonads was achieved by morphological markers. A number of gonads and mesonephros were pooled for RNA extraction. We examined gene expression in the earlier gonads, i.e., E10.5, E11.5, and E12.5, by SAGE and that of the later dates, i.e., E13.5, E15.5, and E17.5 with the Affymetrix oligonucleotide GeneChip microarray. We also used SAGE to examine genes expressed in gonocytes harvested from newborn male mice. We are conducting analyses of the pattern of gene expression at different stages during development and are performing comparative analyses of gene expression in the male and female gonads. We compared the transcriptome of the gonocyte with that of type A spermatogonia as well as with that of E13.5 male gonads to delineate the group of gene clusters that regulate differentiation of primordial germ cells to gonocyte and then to spermatogonia. Results obtained from the studies are expected to yield information important for our understanding of the genetic regulation of gonad development and stem cell differentiation.

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Physiological and genetic effects of disease-causing mutations of the luteinizing hormone receptor

Leung, Baxendale, Wu, Aziz, Pang, Rennert, Chan; in collaboration with Al-Muslim, Fechner, Leschek, Martin

Constitutive activating mutations of the human luteinizing hormone/chorionic gonadotropin receptor (hLHR) cause familial male-limited precocious puberty (FMPP), a noncentral form of gonadotropin-independent precocious puberty. An activating mutation leads to elevated production of testosterone and results in precocious puberty. All the activating mutations of the hLHR resulting in FMPP identified by others as well as by our laboratory are located in the transmembrane domain of the hLHR. Transmembrane helix VI (TMVI) is a particular hot spot of mutation. In a patient with FMPP, we recently identified the novel heterozygous mutation A1738G, which caused the replacement of Thr-580 by Ala. This mutation is downstream of the most frequently found activating mutation Asp578Gly. Our studies of transient transfection and signal transduction via the cAMP pathway as well as the inositol phosphate pathway demonstrated that Thr580Ala did not display the activation phenotype. The influence of the ligands of other G protein–coupled receptors such as FSH and TSH were likewise found to have no effect on signaling in cells expressing the mutated receptor. The results of the study argue for caution in the interpretation of genotype-phenotype correlations in hLHR mutations and FMPP.

In collaboration with Malcolm Martin and Ellen Leschek, we identified two FMPP patients who developed testicular neoplasia. To study the potential tumorigenic effect of a constitutively activated LHR, we have generated an *in vitro* cell model and are in the process of generating a transgenic animal model. We transfected MA-10 cells with LHR carrying activating mutations. Using gene arrays on glass slides containing 22,000 mouse cDNAs, we compared the profile of expressed genes in cells expressing the mutated LHR with that of control cells. Preliminary studies of one mutated LHR indicated up-regulation of genes associated with cell proliferation and down-regulation of genes associated with differentiation. Interestingly, several genes known to be involved in spermatogenesis were also down-regulated in cells expressing the mutated LHR. We plan to compare the profile of gene expression affected by the more common activating mutation Asp578Gly with that affected by the somatic activating mutation Asp578His found in some patients with testicular tumors.

The antithesis of FMPP is Leydig cell hypoplasia (LCH). In LCH patients, mutation inactivates the LHR, resulting in reduced production of testosterone and causing hypergonadotrophic hypogonadism or male pseudohermaphroditism. A novel missense mutation A340T resulting in the substitution of Ile-114 by Phe, which affects one of the six Leu repeats in the extracellular domain of hLHR receptor, has been identified in a patient with LCH. In transient expression studies, the mutant receptor failed to trigger cAMP production upon hCG stimulation. It is known that the mutated LHR, whether activated or inactivated, is abnormally processed by cells. To investigate the trafficking of the mutated LHR *in vitro*, we fused the coding sequence of green fluorescent protein (GFP) to that of wild-type and mutated LHR. We studied the trafficking of the fused protein by fluorescence microscopy. Information generated should further our understanding of the cellular processing of the LHR.

The impact of activating mutation of the LHR has always been considered to be limited to the sexual development of a patient. The abnormal social behavior was thought to be secondary to precocious sexual maturation. Expression of LHR in brain had been demonstrated. We thus speculate that the abnormal behavior of FMPP patients is caused by the expression of the mutated LHR in the brain. To examine this hypothesis, we will identify the cellular location of LHR in the brain. We generated transgene cassettes with an open reading frame encoding a fusion protein of enhanced green fluorescent protein (EGFP) and hLHR mutant with substitution Asp578Gly or Asp578His. The expression of the fusion protein is driven by a 2.1 kb mouse LHR 5′ flanking sequence. We will use the transgene cassettes to generate transgenic mice and then apply the animal model to studying the impact of constitutively activated LHR on spermatogenesis as well as on sexual and neurological development.

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Identification of genetic susceptibility to thrombosis in pseudotumor cerebri

Dogulu, Baxendale, Leung, Chan, Rennert; in collaboration with Kansu, Ozguc

Idiopathic intracranial hypertension (IIH), also known as pseudotumor cerebri (PTC), is a syndrome characterized by symptoms and signs of isolated intracranial hypertension. Even in the absence of clinical, laboratory, or radiological evidence of a space-occupying lesion or hydrocephalus, IIH can lead to catastrophic effects on the visual system. The clinical picture is caused by increased cerebrospinal fluid (CSF) pressure; however, its pathogenesis is not well understood. The most prevalent hypothesis is that the increased CSF pressure is attributable to reduced CSF absorption through the arachnoid villi. Although the majority of cases are nonfamilial, reports of familial PTC raise the possibility of genetic predisposition factors that lead to clinical manifestations following exposure to a precipitating agent. Thus, we hypothesize that IIH is multifactorial and that an underlying genetic thrombotic risk factor predisposes patients to develop local thrombi in the lining of the arachnoid villi, which in turn lead to increased intracranial pressure in the absence of demonstrable cerebral venous thrombosis as judged by conventional imaging techniques. Such a genetic "variation" might occur in coagulation factor V, an enzyme cofactor with pivotal functions in hemostasis. Several polymorphisms/mutations have been identified among the 25 exons of the *Factor V* gene. Altered activity of mutated Factor V is the most common hereditary blood coagulation disorder predisposing to thrombosis. Several mutations/polymorphisms of the *Factor V* gene, including $V_{\text{Hong Kong}}$ (Arg306Gly), $V_{\text{Cambridge}}$ (Arg306Thr), Arg485Lys, V_{Leiden} (Arg506Gln), and the R2 allele (Arg-1299), all known to be associated with thrombotic risk, are located in exons 7, 10, and 13. We scanned the three exons for polymorphisms in 51 IIH patients and 68 controls. The prevalence of three FV thrombosis–associated polymorphisms (Factor V_{Leiden}, 1628 G→A substitution, and R2 allele) in IIH patients was found to be significantly higher than in controls, as was the incidence of associated prothrombotic states. Susceptibility to thrombosis may be an important genetic modifier, which, when present in combination with the other factors, is permissive for development of IIH. Our study is the first report of a genetic polymorphism related to an increased risk of IIH.

Dogulu CF, Kansu T, Leung MYK, Baxendale V, Wu SM, Ozguc M, Chan WY, Rennert OM. Evidence for genetic susceptibility to thrombosis in Idiopathic Intracranial Hypertension. *Thrombosis Res* 2003; in press.

Identification of the role of susceptibility to thrombosis in pseudotumor cerebri of nephropathic cystinosis

Dogulu, Raygada, Chan, Rennert; in collaboration with Gahl, Kaiser

In view of our recent findings regarding genetic susceptibility to thrombosis in PTC in general, we are studying the role of thrombosis susceptibility in the development of PTC in nephropathic cystinosis patients. We are screening nephropathic cystinosis patients who developed PTC and control nephropathic cystinosis patients without PTC by using a thrombosis susceptibility screening panel that includes PT, APTT, Activated Protein C resistance, serum levels of protein C, protein S, antithrombin III, fibrinogen, Factor VIII, Factor IX, Factor XI, total homocysteine, and antiphospholipid antibodies (ACA and Lupus AC). We are also screening for the FV Leiden mutation, FV G1628A polymorphism, FV R2 allele, Prothrombin 20210 mutation, and 5,10-methylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphism in patients with severe homocysteinemia (≥ 100 µmol/l).

Microarray analysis of the tumor suppressor effects of p75^{NTR} and U94 in human prostate cancer

Pang, Rennert, Chan; in collaboration with Ifon, Ravindranath, Rosenthal

Nerve growth factor (NGF) binds to high-affinity tyrosine kinase receptors (TrkA, TrkB, or TrkC) and a low-affinity neurotrophin receptor (p75^{NTR}) on the cell surface. In normal prostate epithelial cells, the major receptors for NGF are the TrkA form of the tyrosine kinase receptor and the p75^{NTR}. During the development of prostate carcinoma, p75^{NTR} was found to be progressively lost. The androgen-refractory human prostate cancer cell line PC3 also lacks the expression of p75^{NTR} while TrkA expression is retained. Reintroduction of p75^{NTR} into PC3 cells decreased the cells' tumorigenic ability in the nude mouse model. Neelakanta Ravindranath hypothesizes that p75^{NTR} acts as a tumor suppressor by specifically regulating the expression of cancer-related genes. To identify the genetic network mediating the tumor suppressor effect of p75^{NTR}, RNA samples isolated from PC3 cells stably expressing p75^{NTR} or transfected with the vector alone were used in microarray hybridizations with human 6K cDNA microarrays to examine the global change in gene expression in the presence of p75^{NTR}. A major portion of the genes showing differential expression patterns in the presence of p75^{NTR} encode signaling proteins, together with growth factors and proteins involved in cell-cell contact.

Leonard Rosenthal and Ekwere Ifon demonstrated that *U94*, a 1437 bp gene of human herpes virus 6A, can drastically inhibit foci formation by PC3 cells *in vitro* and inhibit the tumorigenicity of PC3 cells in the nude mouse model. The molecular mechanism underlying such phenotypic changes remains largely unknown. We used a functional genomic approach similar to that described above to examine the genes that are expressed differentially in the presence of *U94*. Using the human 6K cDNA microarrays, we subjected RNA isolated from the PC3 cells transfected with or without *U94* to cDNA microarray analysis and found that expression of specific sets of genes related to cell-cell contact, proliferation, oncogenes, and growth factor receptors were differentially modulated in the presence of *U94*.

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BRIDGING THE GAP BETWEEN MONOGENIC TRAITS AND COMPLEX INHERITED DISORDERS OF CHILDHOOD

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We focus on several specific areas of biology. Our interests tend to emerge in the context of the infants and children we encounter with certain genetic disorders for which clinical, biochemical, and molecular knowledge is incomplete, for which novel treatment approaches are needed, and for which we believe our patient-oriented studies can be a springboard to advancing understanding in a broader area. Thus, our overarching goal is to improve the understanding, diagnosis, and treatment of these inherited diseases. A longer-term goal is application of genetic approaches to common pediatric diseases for which associated molecular variations will provide the basis both for pathophysiological insights and tailored preventive strategies.

Hemostasis mediated by the platelet glycoprotein (GP)Ibalpha-Ibbeta-V-IX complex

Tang, Liu, Kaler; in collaboration with Steinbach

The platelet membrane glycoprotein (GP)Ib-V-IX complex is the receptor for von Willebrand factor (vWF) and is composed of the four polypeptides GPIbalpha, GPIbbeta, GPIX, and GPV, which all feature leucine-rich repeat motifs. A qualitative or quantitative deficiency in this complex causes the rare human bleeding diathesis Bernard-Soulier syndrome (BSS). BSS is an autosomal recessive trait presenting in infancy with thrombocytopenia, circulating “giant” platelets, and bleeding tendency in infancy. Bleeding in BSS is more severe than predicted by platelet count and is explained by a defect in primary hemostasis. We identified a novel mutation (P96S) at the GPIbbeta locus in an infant haploinsufficient for the gene as a consequence of heterozygous deletion of chromosome 22q11 (velocardiofacial syndrome). Flow cytometry and confocal imaging of transfected Chinese hamster ovary cells that stably express human GPIbalpha and GPIX (CHO alphaIX) on their surface only when transfected with wild-type GPIbbeta demonstrated that P96S GPIbbeta abrogates surface assembly of the platelet vWF receptor complex. Based on amino acid homology to the nogo-66 neuronal receptor (also a leucine-rich repeat protein whose crystal structure has been characterized), we proposed a model of GPIbbeta protein structure (see Figure 9.2) that supports the importance of P96 and six other residues previously reported as missense mutations in the conformation of GPIbbeta and its interaction with GPIX. As the most critical component of this recently characterized platelet adhesion complex, GPIbbeta represents an attractive pharmacologic target for modulating hemostasis.

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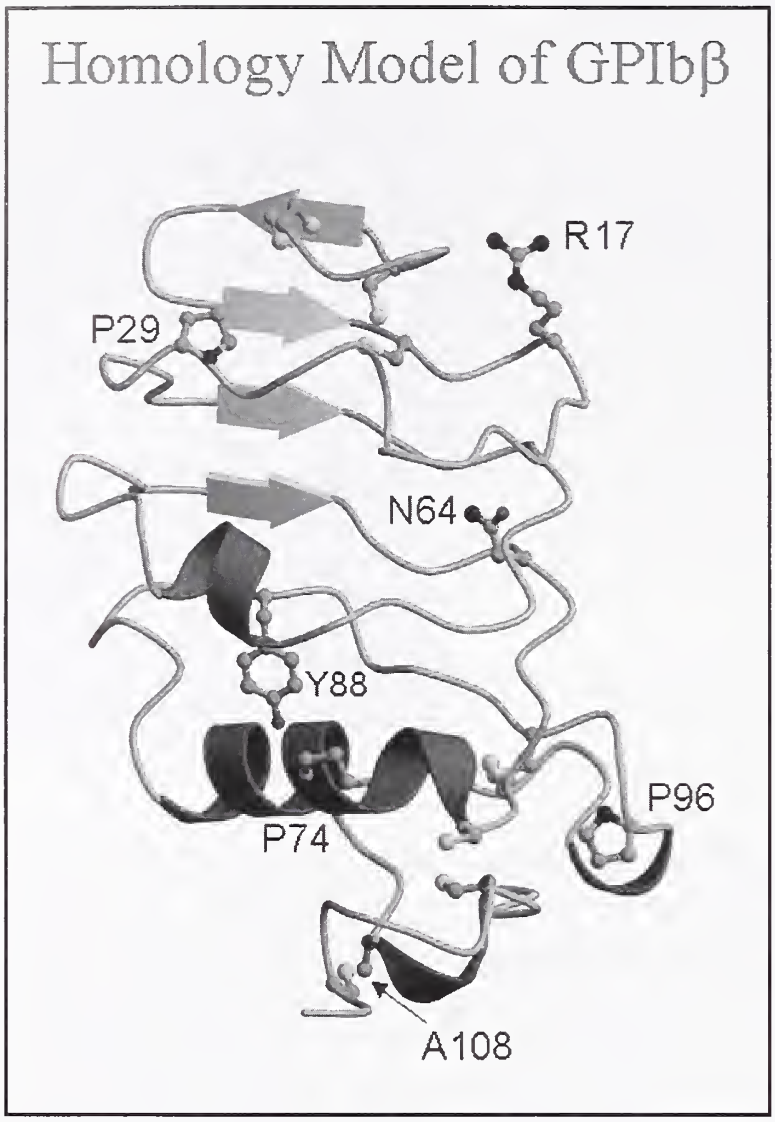

FIGURE 9.2
Model of GPIBbeta

GPIbbeta disorders of copper transport

Liu, Tang, Kaler; in collaboration with Goldstein, Holmes

Menkes' disease is an X-linked recessive neurodegenerative disorder caused by defects in a gene that encodes an evolutionarily conserved copper-transporting ATPase. In mammals, the gene product functions as an intracellular pump to transport copper into trans-Golgi spaces for incorporation into copper-requiring enzymes and to mediate copper exodus from cells. Our work on this disorder includes development of rapid and reliable neurochemical and molecular techniques for very early diagnosis, efforts that dovetail with our clinical trial of very early copper replacement therapy for affected infants. Our results indicate that the blood-brain barrier poses a challenging obstacle in a majority of patients and suggest a molecular basis for treatment responsivity in the minority of patients with successful neurodevelopmental outcomes. Consequently, we are considering alternative therapeutic approaches that bypass the blood-brain barrier.

To unravel the pathophysiological cascades associated with copper deficiency in the developing brain, we age-matched normal controls (accidental deaths) and performed expression profiling studies on post mortem tissues from several of our patients who died despite very early treatment. The patients' mutations predicted little or no functional copper transport activity. As expected, expression of the Menkes' disease gene was detected in control brains but not in the patient tissue. We detected approximately 350 dysregulated genes, which included those involved in neuronal signaling pathways, synaptic function, mitochondrial function, ribosomal function/translation and metabolism, and a number of ESTs of unknown function. The majority of up-regulated genes were involved in anti-apoptosis and stress response. These findings provided an initial insight into the pathogenetic basis of neurodegeneration in Menkes' disease. We have recently extended our studies to the yeast knock-out strain deltaccc2 that lacks the Menkes/Wilson disease gene homolog to determine those genetic effects of copper deficiency that hold from simple eukaryotes to man.

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X chromosome inactivation and developmental anomalies

Tang, Kaler

The constellation of birth defects sternal cleft, abdominal raphe, and hemangiomas shows a distinctive female predilection; available medical literature indicates that nearly all cases (> 92 percent) of this syndrome occur in females. This situation is also seen in the related phenotype PHACE (*p*osterior fossa brain malformations, *h*emangiomas, *a*rterial anomalies, *c*oarctation of the aorta and *c*ardiac defects and *e*ye abnormalities). Nonrandom (or “skewed”) X-chromosome inactivation has been implicated in the etiology of certain X-linked dominant traits. In such situations, female carriers of deleterious alleles on one X chromosome are spared disease manifestations due to favorably skewed X inactivation patterns; however, their female offspring (in whom X inactivation is random) are at risk for expression of the mutant allele. Prenatal lethality in male offspring who inherit the mutant allele explains the observed female predominance. We documented skewed X inactivation in the mother of a PHACE patient and speculate that the phenotype represents an X-linked dominant trait that is lethal in males. We are exploring the hypothesis that defects in a transcription factor or other X chromosomal gene influencing development is responsible for the PHACE phenotype.

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CHILD AND FAMILY DEVELOPMENT IN THE FIRST TWO DECADES OF LIFE

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We investigate dispositional, experiential, and environmental factors that contribute to physical, mental, emotional, and social development in human beings during the first two decades of life course. Our overall goals are to describe, analyze, and assess the capabilities and proclivities of developing children, including their genetic characteristics, physiological functioning, perceptual and cognitive abilities, and emotional, social, and interactional styles as well as the nature of and consequences for children and parents of family development and children's exposure to and interactions with their physical surroundings. Project designs are experimental, longitudinal, and cross-sectional as well as intra- and cross-cultural. Sociodemographic comparisons include family socioeconomic status, maternal age and employment status, and child parity and daycare experience. In addition to the United States, study sites include Argentina, Australia, Belgium, Brazil, Cameroon, Canada, England, France, Israel, Italy, Japan, Kenya, Peru, and the Republic of South Korea.

Parenting and child development

Bornstein, Hahn, Haynes, Hendricks, Leach, Painter, Suwalsky

We are broadly concerned with analyzing and understanding the roles of parenting in human development. Methodological assessments of parenting show sometimes similar, sometimes different patterns of findings reflective of different approaches to the same problem. In one study, we compared frequencies of behaviors of mothers and infants in two cultures based on continuous coding with frequencies based on time-sampling and evaluated the resulting patterns yielding different estimates of absolute frequency of typical maternal and infant behaviors between individuals and between cultural groups. However, time-sampling adequately preserved the relative ranking of mother and infant behaviors among individuals and between cultural groups. If research is concerned with the effect of relative standing of individuals and/or groups on frequency of maternal or infant behaviors, under specified circumstances time-sampling and continuous coding yield comparable results.

We investigated parenting knowledge in mothers of young children, where mothers obtain their knowledge, and what factors principally influence the amount and accuracy of that knowledge. U.S. mothers of two-year-olds completed a survey of parenting knowledge and sociodemographic and health status variables as well as their sources of support for parenting. Japanese American (JA) and South American (SA) mothers acculturating to the U.S. gave the same information, as did mothers representing six additional countries (Argentina, Belgium, France, Israel, Italy, and Japan). U.S. mothers scored well on the evaluation of parenting knowledge; mothers' age, education, and access to written materials were each uniquely associated with higher scores. We found no differences between mothers of girls and boys, between birth and adoptive mothers, or in effects of mothers' employment status; however, adult mothers scored higher than adolescent mothers, and mothers improved in their knowledge of parenting with time. Acculturating mothers scored less well than U.S. mothers but better than mothers from their cultures of origin; U.S., Israeli, and Belgian mothers tended to score highest. Written materials and attendance in childbirth classes contributed to parenting knowledge in non–U.S. groups but the contributions of fathers and friends/neighbors emerged as significant. Understanding variation in parenting knowledge and its sources has implications for parenting education as well as for pediatrician training and interactions with parents.

We also examined the roles of multiple contributors to variation in key maternal perceptions of their own parenting. In separate predictions of self-perceived competence, satisfaction, investment, and role balance in European American mothers of first-born 20-month-olds, we explored maternal socioeconomic status (SES), employment, parenting support, child gender, language and social competence, temperament, maternal intelligence, personality, and parenting knowledge and style. Hierarchical regression analyses supported highly differentiated patterns of unique predictive relations to each domain of self-perceived parenting. Nonetheless, some predictors consistently contributed to individual parenting self-perceptions, most prominently parenting knowledge and dissonance between actual and ideal maternal and parental parenting styles. SES, maternal employment, community support, and maternal personality also contributed to self-perceptions, as did child temperament. Although the potential contributors to parenting self-perceptions may be several, prominent contributors to any one self-perception are specific and few, a conclusion that articulates with the modular view of parenting.

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Family and child acculturation in modern America

*Bornstein, Cote**

Current census statistics indicate that one out of every five children under the age of 18, or 14 million children, in the United States are either immigrants themselves or the children of immigrant parents. Yet the nature of acculturation itself is elusive and remains poorly understood; moreover, acculturation is a major transforming force on child health and human development. We study families acculturating to the United States from Japan and South America, assessing the role of acculturation on parenting, child development, and family life.

One study investigated parenting beliefs among mothers from these countries. First, we undertook a longitudinal evaluation of cultural differences and developmental continuity and stability in cultural cognitions (acculturation, individualism, collectivism) and parenting cognitions (attributions, self-perceptions, knowledge) when children were five and 20 months of age. SA mothers were more collectivistic than JA mothers. Cultural group and attribution differences emerged for mothers' parenting attributions in successful situations, whereas child age and attribution differences emerged for parenting attributions in unsuccessful situations. Specifically, SA mothers rated ability and task attributions more strongly than JA mothers in success situations. In unsuccessful situations, mothers rated child behavior as a cause when their children were 20 months of age more than when they were five months of age. JA mothers' feelings of competence increased over time. SA mothers were more satisfied in the parenting role than JA mothers. Mothers' knowledge of parenting increased over time in both groups. Mothers' cultural cognitions were largely stable, as were JA mothers' parenting cognitions. The finding that parenting cognitions remained largely stable, despite major developmental changes in the child being parented, suggests that parenting beliefs may be adopted from the culture rather than formed on the job. The study provides insight into the differential influence of cultural background on the acculturation of cultural and parenting cognitions in two U.S. acculturating groups.

In a second study, we explored prediction and coherence in cultural (acculturation, individualism, collectivism) and parenting cognitions (attributions, self-perceptions, knowledge) in JA and SA mothers. Mothers' cultural cognitions when their infants were five months old predicted some parenting cognitions 15 months later, particularly among JA mothers. Specifically, JA mothers' acculturation level positively predicted their knowledge of U.S. parenting norms, and their level of collectivism positively predicted their effort attributions and satisfaction with parenting. SA mothers' collectivism negatively predicted their parenting knowledge. Coherence among attributions both within and across parenting situations at both time periods and for both cultural groups suggests that the mothers possess attributional styles, with some mothers strongly looking for multiple reasons for their behavior and others less prone to look for reasons for their behavior, particularly among SA mothers. The study also found coherence among JA mothers' self-perceptions of parenting at both time periods, particularly with respect to competence and satisfaction, suggesting that these are important interrelated factors in JA mothers' perceptions of how they handle the parenting role, at least when their children are young. The study results showed few relations between mothers' attributions and their self-perceptions or knowledge at either five or 20 months, suggesting that attributions are relatively independent of self-perceptions and knowledge.

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Infant and child development

Bornstein, Mash; in collaboration with Arterberry

The capacity to categorize objects, events, and other aspects of experience lies at the core of adaptive, intelligent behavior. In this context, categorization refers to the treatment of discriminable entities as equivalent in some way. Without this ability, every distinct encounter with the environment would demand a unique response, a demand that would quickly exceed human capability. When treating similar entities as equivalent, humans can store functionally relevant information about each entity in a unified manner instead of storing entities redundantly across each instance. Furthermore, accessing the representations that are associated with a given category can furnish information about completely novel entities as soon as those entities are categorized. Thus, categorization not only provides an economical manner in which to organize knowledge and skill but also allows us to extend that knowledge and skill beyond the limits of our own direct experience and consequently has far-reaching adaptive significance. Accumuled evidence now indicates that human infants are surprisingly capable of formulating and representing categories of visual information.

We investigated variability in infants' categorization performance and potential sources of variability. Using data from 13 categorization studies employing a habituation-of-looking paradigm with infants three, five, six, and nine months of age, we developed a method for establishing a categorization criterion for use in classifying individual infants as "categorizers" for particular tasks and used logistic regression analyses to identify demographic and information-processing variables that predicted "categorizer" classification. Variables that increased the odds of classification as a categorizer were gender, number of habituation trials, and duration of peak look during habituation; total looking time during habituation decreased the odds of categorizer classification. Our findings have implications for individual differences in information processing.

We investigated infants' categorization of animals and vehicles based on static versus dynamic attributes of stimuli. Six-month-olds categorized static color images of animals and vehicles and dynamic point-light displays showing only motions of the same animals and vehicles. In other experiments, three-, four-, six-, and nine-month-olds were tested in a habituation-transfer paradigm. Half the infants at each age were habituated to static images and tested with dynamic point-light displays; the other half were habituated to dynamic point-light displays and tested with static images. Six-month-olds did not transfer. Only nine-month-olds who were habituated to dynamic displays showed evidence of category transfer to static images. This study highlights age effects in categorization and the relative importance of static and dynamic stimulus information for categorization.

Perceiving emotions correctly is foundational to the development of interpersonal skills. We tested five-month-old infants' abilities to recognize, discriminate, and categorize facial expressions of smiling. Infants were habituated to four degrees of smiling modeled by the same or different people; following habituation, infants were presented with a new degree of smile worn by the same and by a new person, a new degree of smile and a fearful expression worn by the same person, or a new degree of smile and a fearful expression worn by new people. Infants showed significant novelty preferences for the new person smiling and for the fearful expressions over the new degree of smiling. These findings indicate that infants at five months categorize the facial expression of smiling in static faces yet recognize the same person despite changes in facial expression; five months is the youngest age at which these categorization abilities have been demonstrated. The findings have significance for face processing in social interaction and infants' categorization of faces.

We further examined categorization in young children using a sequential touching procedure. We assessed 12-, 18-, 24-, and 30-month-olds' categorization at superordinate, basic, and subordinate levels in four object domains (animals, vehicles, fruit, furniture) that contrasted living with nonliving objects and stationary with moving objects. Superordinate categorization was uniformly good across these ages; a basic level categorization emerged, first for high perceptual contrasts and later for low perceptual contrasts; and subordinate categorization was uniformly absent across all ages. A developmental advantage for categorization of living things emerged. In another study, 20-month-olds' categorization was replicated and explored in greater depth in six object domains (animals, vehicles, fruit, furniture, tools, dishes). Domain sets also contrasted category level and relevant versus irrelevant perceptual features. Twenty-month-olds categorized at the superordinate and basic levels but not at the subordinate level; they categorized multiple superordinate domains; and they ignored irrelevant perceptual attribute information, such as size and color, when categorizing.

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CHILD DEVELOPMENT IN CONTEXT

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Our research is designed to explore how developmental processes are influenced by their social and physical context. To elucidate these influences and processes, researchers must examine the interface between endogenous and exogenous processes, children's conceptions and perceptions of their experiences, and the ways in which knowledge of developmental processes can inform social policies and practices.

Facilitating children's accounts of experienced events
Lamb, Abbott, Orbach, Pipe, Sternberg, Guterman, Thierry; in collaboration with Aldridge, Brown, Cederborg, Esplin, Hershkowitz, Horowitz, Lewis
One major program of research has involved the development and assessment of techniques for enhancing the informativeness of child witnesses and for evaluating the credibility of their accounts. Most of our studies focus on the relationship between interviewer style and the quality of information provided by young children, confirming that open-ended questions elicit longer and more detailed responses than more focused questions. Information elicited in response to open-ended prompts of recall memory is also more likely to be accurate in both analog and forensic contexts. Such findings have strengthened the generalizability of the results obtained in many laboratory studies.

In research conducted in collaboration with investigative agencies in the United States, United Kingdom, and Israel, we have shown that, regardless of children's ages, interviewers can increase the length and richness of children's accounts by following protocols we designed to probe recall memory and reduce the reliance on more focused questions, which are more likely to elicit erroneous information. Use of the NICHD Investigative Interview Protocol dramatically increases the amount of information retrieved from four- to 13-year-old alleged victims responding to open-ended prompts. Although preschoolers are often deemed incapable of responding to open-ended prompts, our research shows that a similar proportion of details can be elicited by using open-ended prompts from children as young as four years and as old as 13 years. There are, however, important age differences in the types of information that children provide. For example, ongoing analyses of interviews show steady increases with age in the amount and quality of information children provide about the timing of events. This research has provided unique insight into children's developing appreciation of temporal information about experienced events, whereas most research on the understanding of time has involved laboratory research.

Adaptations of the NICHD Investigative Interview Protocol for use with young witnesses and alleged perpetrators are currently being used in the field as well. Our research has shown that young witnesses recall as much information in total, as well as in response to open-ended prompts, as do alleged victims. Suspects tend to be more reluctant, but those who agree to talk provide as much information about their experiences as age mates who are alleged victims. Other field experiments have shown that mental context reinstatement and the introduction of gender-neutral anatomical drawings in the context of protocol-guided interviews also help children provide substantial numbers of additional details about alleged incidents of abuse. The gender-neutral drawings were especially productive when shown to young (four- to seven-year-old) children. Because we do not know whether the additional information is accurate, we are currently initiating research designed to assess the accuracy of reports provided by young children about both staged experienced events and fictitious events.

In other ongoing research, we are exploring the extent to which use of the protocol facilitates decisions and interventions designed to prosecute offenders and protect children. Given, however, that many children do not disclose suspected abuse when interviewed, we are also exploring the characteristics of cases in which children do and do not make allegations or make allegations only reluctantly. We hope to understand the factors that lead children not to report abuse that they actually experienced. These studies, too, should help us develop procedures that can be implemented nonsuggestively in forensic settings in order to enhance the sensitivity and specificity of conclusion drawn from investigative interviews.

Another program of research is concerned with the effects of child and spouse abuse on the development of children and adolescents. Independent interviews with mothers, fathers, and children revealed widely divergent accounts of the families' histories of violence. The differences complicate efforts to identify links between experiences and outcomes. In both middle childhood and adolescence, however, family violence appears to affect the offsprings' views of their parents. Children/adolescents felt less closely attached to the parents who had abused them, whereas spouse abuse had little apparent effect on the children's attachments to their parents. More recent family experiences had clearer effects on the children's behavior problems and perceptions of their parents than did earlier experiences.

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Adaptation to nonparental child care

Lamb, Abbott, Chuang; in collaboration with Ahnert, Hwang

Another program of research has involved short- and long-term longitudinal studies in Göteberg (Sweden) and Berlin (Germany) of children with distinct child care experiences. The longitudinal study in Sweden was initially designed to elucidate the effects of early care arrangements on the development of 145 children recruited in 1982 at an average age of 16 months. Initial analyses indicated that the quality of home care and the quality of alternative care had substantial effects on the children's verbal abilities, social skills, and personal maturity. The effects diminished as the children moved into the formal educational system and their individual personalities came to affect the adjustment to school. Preliminary analyses revealed no apparent effects of contrasting early care patterns on educational histories and the psychological status of these children at 15 years of age.

Longitudinal analyses revealed substantial stability over time in the children's personality styles. Of the Big Five personality factors, conscientiousness was coherent from toddlerhood, whereas the internal reliabilities of extraversion, agreeableness, neuroticism, and openness to experience increased over time. Scores on most of these factors were fairly stable over time, but children became less extraverted, more agreeable, and more conscientious with age. Although individual differences were stable, the children also became more ego-controlled over time. Boys' levels of ego resiliency were more stable over time than girls'; boys became less resilient from middle childhood into mid adolescence, whereas girls became more ego-resilient as they entered adolescence. Participants in the study are being reinterviewed in 2003–2004, when they average 21 years of age.

In the Berlin longitudinal study, we have been measuring the psychophysiological, socioemotional, and behavioral tendencies of infants so that we can assess the effects of earlier individual differences in behavioral and psychophysiological reactivity and infant-mother attachment on the adaptation to out-of-home center care. During an adaptation phase, in which mothers remained in the centers with their toddlers, secure infants had markedly lower cortisol levels than insecure infants, suggesting that they gained more protective support from the presence of their mothers. When the mothers stopped remaining with their infants, the cortisol responses of the securely attached toddlers were much more dramatic than the responses of the insecurely attached toddlers: on the initial separation days, cortisol levels rose over the first 60 minutes after arrival to levels twice as high as those at home. Secure toddlers also fussed/cried upon separation more than insecurely attached toddlers. Cortisol and behavioral markers of distress were correlated in securely attached but not in insecurely attached toddlers. The security of attachment changed in many cases following the onset of child care, but attachments were more likely to become or remain secure when mothers remained longer in the child care facilities with their toddlers. Close examination of individual differences in cardiac reactivity and of the formation of relationships with care providers are now under way.

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Subcultural variations in the nature of children's early experiences

Lamb, Fouts; in collaboration with Ahnert, Hewlett, Roopnarine

Another project has focused on the description of cultural differences in early interaction. In each case, we conducted extended observations to ensure the reliable measurement of individual differences. To explore further the effects of culture and context on early interactions, we are conducting comparable day-long observations of parents and infants in Quebec, Germany, the Central African Republic, Costa Rica, and Colombia as well as in African American, Caribbean American, and Euro-American families in the United States.

Extended observations of two- to five-year-old Bofi infants whose families either lived in villages or pursued a nomadic hunting and gathering lifestyle revealed that, contrary to widespread beliefs, weaning was seldom a time of parent-offspring conflict. In both groups, children initiated weaning as they began to eat a variety of foodstuffs, although villagers were more likely to terminate breastfeeding at a predetermined time, whereas foragers let the children themselves set the pace. Weaning was usually associated with pregnancy, and the children's reactions varied depending on the availability of additional care providers as well as on maternal sensitivity.

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Subcultural variations in parental and filial perceptions and beliefs

Lamb, Chuang, Fouts; in collaboration with Cabrera, Hwang

We have also been investigating ways in which variations among rearing environments (especially as indexed by parental beliefs, values, and practices) affect children's development. In one line of research, we are assessing longitudinally the gender differences in the self-perceptions of two cohorts of seventh to 12th graders so that the antecedents and correlates of different styles of self-perception in adolescence can be explored. Rating themselves in 11 different roles, girls perceived themselves as more affiliative and less negatively affiliative in many roles than did boys, but gender differences in assertion were not reliable and girls' assertiveness did not decline over time. The results contrast with popular claims regarding girls' "loss of voice" in adolescence. Gender differences were context-specific and were more pronounced in ratings of Myself as a boy/girl and Myself with a close same-sex peer. To explore antecedents of these gender differences further, we gave portions of the self-perception battery to a group of Swedish 15-year-olds whose development has been documented systematically since infancy. Analyses of the data are currently under way, but preliminary results suggest few reliable correlates of individual differences in the Swedish sample.

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MECHANISMS REGULATING AUDITORY COMMUNICATION IN PRIMATES

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Understanding the mechanisms underlying the expression and perception of auditory communication in nonhuman primates provides important insights into the neural systems that mediate nonverbal auditory communication in humans. Our research is devoted to investigating the changes in vocal behavior that are associated with maturation and social experience under normative conditions as well as the neural systems implicated in such behavior so that we can define their roles in mediating auditory communication. Vocal development is a dynamic process, and a pattern shared by several nonhuman primates has emerged regarding the nature of this process. Infants are highly vocal during periods of brief separation from their caregiver, and we take advantage of this behavior to document the range of vocalizations produced by infants of different ages. The role of individual experience during development is currently under investigation along with that of putative neural substrates to determine the mechanisms leading to the acquisition of adult vocal skills.

Acoustic dimensions underlying species-specific vocal communication

Newman, Soltis, Bernhards, Sander

Adult squirrel monkeys make characteristic cry-like sounds when separated from their social group as well as upon hearing other individuals making the same sounds. Two species of squirrel monkey in our colony make cry sounds that are species-specific in their acoustic details, and some evidence from preliminary studies along with a published study using phonotaxic responses to infant cries suggests that each species preferentially responds to its own species' cries over those of the other species. The present study used responsive calling as an index of selectivity to digital samples of the cries of adults of both species. Animals were tested in their social groups, as were individual vocalizers' responses to each playback stimulus identified by human observers familiar to the subjects. Stimuli were cries of both species randomly ordered in groups of 16 trials per day. Subjects were tested in outdoor enclosures, where they would occasionally hear (and respond to) the cry sounds of other squirrel monkey groups at our facility. Stimuli were presented during silent periods of at least two minutes. Two groups of one of the two species have been tested to date. Individuals of these groups responded to cry stimuli from their own and the other species, invalidating the hypothesis that each species would only respond to its own species' cries. Two groups of the second species will be tested.

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Neuroethology of crying

Newman, Boyd, Bernhards, Sander

The primate isolation call is functionally and acoustically homologous with the cry sounds of human infants. Like human infants, nonhuman primate infants emit continuous cry sounds when separated from their caregiver and social group. Given the difficulty of conducting invasive experimental neurological procedures in infants, little is known about the neural substrates underlying crying. However, the activation of the *c-fos* gene has been shown in a variety of behavioral contexts to identify brain areas involved in production of the behavior under study. The peptide product of *c-fos* activation, Fos, can be detected by immunocytological methods. Hence, the present study aims to identify Fos-labelled neurons following terminal experiments in which the animal is separated, allowed to make cry sounds for 20 to 60 minutes, and euthanized; we then process the brain for Fos immunocytochemistry. To date, we have studied infant common marmosets one to five months of age and have found label in areas expected to be involved in vocal production (hypothalamus, central gray of the midbrain) as well as in areas not thought to be involved in vocal production. In addition, a structure known to be involved in making the adult form of the species-typical cry sounds, the anterior cingulate gyrus, has shown little sign of Fos labeling. Most of the animals used so far have been suboptimal due to their weak crying (a result of poor health or other factors), and further work will use infant marmosets with more robust crying behavior and a broader age range.

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Maternal recognition of own infant's cries

Newman; in collaboration with Kose, Lorberbaum

In earlier work on this project using fMRI methods and human infant cries as acoustic stimuli, functional scans of mothers revealed several brain areas activated by the cries but less so or not at all by artificial noises. In the present study, we recruited a new sample of mothers and recorded the mothers' own infant cries for use in the fMRI scans. Infants six to eight weeks postpartum were exposed to a paradigm previously used to elicit cries only in neonates. The infant was undressed and placed on a cool surface two hours after feeding. Cries were induced in 33 to 34 infants within five minutes of the start of the procedure. Mothers (along with some fathers) were later asked to distinguish a digital recording of their own infant's cry sounds from the cry sounds of a healthy eight-week-old female infant used as a "standard" cry. All parents correctly identified their own infant's cry sounds. Parents also rated the cry from their own infant more like the cry sounds they heard at home than the cry of the unfamiliar infant. Parent subjects are now undergoing fMRI scans by using the same pair of acoustic stimuli (own infant's cry and standard cry) to examine whether new brain areas are activated beyond those activated in our earlier work using only standard cry stimuli.

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GENETIC AND ENVIRONMENTAL DETERMINANTS OF BIOBEHAVIORAL DEVELOPMENT

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Our work involves broad-based investigation of primate biobehavioral development through comparative longitudinal studies of rhesus monkeys and other nonhuman primate species. Our primary research goals are to characterize distinctive biobehavioral phenotypes in our rhesus monkey colony, to determine how genetic and environmental factors interact to shape these primates' development, and to assess the long-term biobehavioral consequences for monkeys from different genetic backgrounds when they are reared in various physical and social environments. A second major program of research investigates how rhesus monkeys and other nonhuman primate species born and raised under different laboratory conditions adapt to placement into environments that model specific features of their natural habitat. We assess adaptation by examining behavioral repertoires and monitoring a variety of physiological systems in monkeys throughout the lifespan, yielding broad-based indices of relative physical and psychological well-being. In similar fashion, we assess the responses of subjects to experimental manipulations of features of their environment. Whenever possible, we collect field data for appropriate comparisons. We also focus on investigating the cognitive, behavioral, and social processes involved in adaptation to new settings and circumstances.

Developmental continuity of individual differences in rhesus monkey biobehavioral development

Anton, Higley, Novak, Parker, Robbins, Roma, Shannon-Lindell, Turrio, Suomi; in collaboration with Lopez

Several studies focus on interactions between a polymorphism (due to length variation in the promoter region) in *5-HTT*, a candidate gene for impaired serotonergic function, and differential early social experience. This past year, we reported a specific gene-environment interaction in rhesus monkeys during their first month of life: compared with those with the "long" (LL), allele infants with the "short" (LS) allele exhibited deficits in measures of state control and visual orienting, but only if they had been nursery-reared; in contrast, LS monkeys reared by their biological mother showed normal state control and visual orienting, suggesting a "buffering" effect of maternal rearing. The ACTH response to social separation in late infancy of LS rhesus monkeys, the expression of impulsive aggression and social play behavior during the juvenile years, and the propensity to consume alcohol in a "happy hour" situation during adolescence provided additional examples of maternal buffering.

A parallel pattern of gene-environment interaction involving a polymorphism in the *MAO-A* gene was found for levels of aggressive behavior exhibited by mother- and peer-reared rhesus monkey juveniles. This past year, we also identified and characterized additional polymorphisms in the *CRH* gene and in the *Neuropeptide Y* gene as well as a single nucleotide polymorphism (SNP) in the dopamine transporter gene and are now determining whether these polymorphisms are associated with specific gene-environment interactions with respect to a variety of behavioral and biological measures obtained.

Rhesus monkeys are notoriously aggressive as a species compared with other macaques (indeed, compared with most other primates); by contrast, Barbary macaques are unusually nonaggressive. Last year, we genotyped members of a group of free-ranging Barbary macaques with respect to the *5-HTT* gene and, unlike the case of rhesus monkeys, we found no individuals with either the LS or the LL allele. Instead, all the sampled Barbary macaques had an "extra long" (XL) allele, a form found in less than 2 percent of the rhesus monkeys genotyped to date. We subsequently genotyped additional Barbary macaques that came from other geographic regions and replicated our initial findings, suggesting that the XL allele is indeed representative of the species as a whole rather than reflecting a specific founder effect. We also genotyped the different Barbary macaque populations with respect to the *MAO-A* gene and once again failed to find the polymorphism previously seen in our rhesus monkey population. We are now genotyping both the *5-HTT* gene and the *MAO-A* gene in three other species of macaques and are characterizing the *5-HTT* gene in a large number of other nonhuman primate species representing the full range of taxa across the entire primate order as a first step in modeling the evolutionary history of 5-HTT in primates.

This past year, we published our findings that intracebroventricular (ICV) infusions of CRH were associated with (1) increases in both anxiety-like and observer-directed behaviors in young adult rhesus monkeys housed both individually and in familiar social groups and (2) increases in depressive-like behaviors in the social group setting. Such infusions were also associated with significantly increased glucose metabolism in amygdala, pituitary/infundibulum, and hippocampus, indicating enhanced limbic brain activity, while administration of the CRH-R1 receptor antagonist antalarmin not only decreased binding in brain regions with high CRH-R1 concentrations but also significantly decreased 5HT-2A binding in right posterior temporal cortex, thalamus, and left and right striatum and slightly decreased the binding in the left and right anterior temporal cortex, left and right orbital cortex, and left and right dorsolateral prefrontal cortex, thus linking HPA activity and serotonergic function in the primate brain.

Post mortem studies of mother- and peer-reared young adult rhesus monkeys revealed significant differences in both brain structure and function as a result of differential early rearing history. Compared with their mothers, peer-reared monkeys exhibited significantly lower mineralocorticoid recepter (MR) mRNA levels in hippocampus but, somewhat paradoxically, significantly higher MR mRNA levels in prefrontal cortex yet significantly lower 5-HT1A mRNA levels and less 5-HTT binding. Furthermore, peer-reared monkeys with the LS 5-HTT polymorphism had significantly lower 5-HT1A mRNA levels and less 5-HTT binding than those with the LL polymorphism, whereas there were no significant differences between LS and LL mother-reared subjects in either measure, thus demonstrating a gene-environment interaction at the level of brain structure and function.

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Adaptation of laboratory-reared monkeys to field environments

Kerekes, Miller, Morin, O'Neill Wagner, Roma, Shannon-Lindell, Suomi

This past year, we completed data collection for a study in which some nursery-reared rhesus monkey infants ("masters") were given operant control over access to highly desirable food treats, whereas other nursery-reared infants ("yoked controls") received the same treats in the absence of any control. Preliminary analyses of behavioral data collected in both the infants' home cages and a novel environment indicated that the "master" subjects engaged in more exploratory and less anxious-like behavior and had lower levels of HPA activity than their yoked control counterparts. Additional analyses of CSF monoamine metabolite concentrations are currently under way.

A second study completed this past year examined the effects of increasing the amount of physical handling of nursery-reared rhesus monkey infants by our nursery staff on behavioral development, HPA activity, and CSF monoamine metabolite concentrations. Throughout their first 139 days of life, one group of nursery-reared infants received an "extra" 32 minutes of daily handling over that typically experienced by rhesus monkey infants reared in the LCE neonatal nursery. The infants receiving the extra physical handling consistently exhibited significantly lower levels of ACTH when tested under standardized conditions, beginning at 30 days of age and continuing beyond the period of extra handling, relative not only to their nursery-reared counterparts receiving standard nursery care but also compared with same-age infants reared by their biological mothers. Analyses of the behavioral and CSF monoamine metabolite data are currently under way.

We developed and pilot-tested two new methodologies over the past year. The first involved the development of an apparatus named the Saliva Procurement Integrative Tool (SPIT) for repeated noninvasive collection of saliva samples from several nursery-reared rhesus monkey infants. The device is constructed of inexpensive, readily available materials and allows investigators to sample multiple infants simultaneously without being present during saliva collection. The second methodology used small accelerometers housed inside wristwatch-sized cases placed within modified commercially available small nylon pet harnesses carried on the backs of nursery-reared infants housed both individually and in small social groups. The accelerometers monitored the physical activity in each infant every 15 minutes over several 96-hour periods throughout development. Data from an initial study monitoring the activity levels of differentially reared infants during their fourth and fifth months of life are currently under analysis.

Another study explored the relationship between adrenocortical reactivity and object manipulation style measured in infant, juvenile, and adult capuchin monkeys and object manipulation style and proficiency in a tool-using task assessed four years later. Individual differences in object manipulation style among monkeys of all ages were predictive of individual differences in both object manipulation style and successful tool use later in life. Among infants and juveniles, manipulation and success in both the initial and subsequent tool-using tests were negatively correlated with plasma cortisol concentrations; adults showed no significant correlations between tool-using scores and cortisol reactivity.

This past year, we published the results of a study examining continuity and change in patterns of adrenocortical functioning throughout the juvenile years in rhesus monkeys reared in three different physical and social environments for the first seven months of life but thereafter placed together and subsequently maintained in large peer groups until puberty. Monkeys from all three rearing conditions (mother-peer, surrogate-peer, and peer-only) exhibited significant increases in plasma cortisol over their first five months but then showed steady declines from one to three years of age;

females consistently had higher concentrations than males at all ages. Plasma cortisol levels of surrogate-peer–reared subjects were lower than those of their mother-peer–reared counterparts (with peer-only–reared subjects intermediate) during the first month of life but not at later ages. Juvenile cortisol levels were significantly correlated with infant levels in all samples collected after two weeks of age, indicating long-term developmental stability of individual differences. Saliva samples were collected from a subset of subjects at one, two, and three years, revealing a significant age-related decline in salivary cortisol concentrations as well as lower levels for surrogate-peer–reared monkeys at each age, but no significant sex differences at any age. Comparison of salivary and plasma cortisol values obtained from the same subjects yielded a highly significant positive correlation.

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CONJUGATE VACCINES AGAINST ENTERIC PATHOGENS

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Surface polysaccharides of Gram-negative enteric pathogens, in the form of capsule or lipopolysaccharide, are both essential virulence factors and protective antigens. We enhanced the immunogenicity of these polysaccharides by binding them to carrier proteins.

Salmonella typhi
Szu; in collaboration with Bystricky
Sequential clinical studies in adults and in children in Vietnam, an area with a high attack rate of typhoid fever, showed that the capsular polysaccharide (Vi) of *Salmonella typhi* conjugated to the recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA) elicited high responses against *S. typhi*. In a Phase III trial, about 12,000 two- to five-year-old children injected with the conjugate vaccine showed no significant side reactions. The efficacy of Vi-rEPA was 91 percent after 27 months of active surveillance and 89 percent after 48 months of surveillance. Children in the placebo group received the conjugate vaccine two and half years later.

Szu SC, Bystricky S. Physical, chemical, antigenic, and immunologic characterization of polygalacturonan, its derivatives, and Vi antigen from Salmonella typhi. *Methods Enzymol* 2003;363:552-567.

Salmonella paratyphi A
Salmonella paratyphi A is the second most common cause of enteric fever in Southeast Asia and, in Southeast China, its incidence exceeds that of typhoid. A conjugate vaccine against *S. paratyphi A* was found to be safe and immunogenic in adults, teenagers, and then in two- to four-year-old children; a Phase III clinical trial is planned.

Escherichia coli O157
Escherichia coli O157, an emerging pathogen, causes hemolytic uremic syndrome in young children. A Phase I study of an O157 O-specific polysaccharide–rEPA conjugate vaccine demonstrated safety and immunogenicity in adult volunteers. A Phase II study, in which 52 two- to five-year-old children were recruited and injected once or twice, showed that the vaccine was safe. Preliminary immunogenicity data showed that children responded uniformly with greater than 10-fold rise of anti–LPS IgG six weeks after the first injection. To produce a bivalent vaccine, we will conjugate a nontoxic shiga toxin I purified from mutant *E. coli* O157 with O-specific polysaccharide. The

major reservoir of *E. coli* O157 is cattle, in which an LPS-protein conjugate was shown to be immunogenic. A challenge study showed an inverse correlation between the serum anti–LPS IgG and the level of rectal colonization of *E. coli* O157.

Vibrio cholerae O1 and O139

Szu, Bryla, Robbins; in collaboration with Chu, Schiloach, Schneerson

Vibrio cholerae O1 and O139 are the major serotypes in cholera infections. Conjugates synthesized with capsular polysaccharide of O139 and O-specific polysaccharide of LPS elicited vibriocidal antibodies in mice. Clinical trials of these conjugates are planned. Detoxified LPS from both Inaba and Ogawa serotypes have been used as a base for conjugate vaccine. In animal studies, the conjugates elicited higher antibody levels than LPS alone. A chemically synthesized O-specific polysaccharide for Ogawa will be prepared and conjugated to protein carrier. We will compare the immunogenicities of cholera vaccines prepared from naturally purified O-specific polysaccharide with those prepared from a chemically synthesized O-specific polysaccharide. We plan clinical trials of these conjugates.

Mai NL, Phan VB, Vo AH, Tran CT, Lin FY, Bryla DA, Chu C, Schiloach J, Robbins JB, Schneerson R, Szu SC. Persistent efficacy of Vi conjugate vaccine against typhoid fever in young children. *N Engl J Med* 2003;349:1390-1391.

Salmonella typhimurium

Salmonella typhimurium is the most common cause of salmonellosis in developed countries. Antibiotic-resistant strains have become common in disease isolates. We have compared the O-specific polysaccharide structure of the most common and antibiotic-resistant strains. The O-acetyl group on the abequose was found to be a major antigenic site and an immunodominant determinant. O-acetyl–positive strains reacted poorly with antibodies from O-acetyl–negative strains. Selection of strains for vaccine preparation will therefore rely on an epidemiology survey and bacteriologic identification.

Campylobacter jejuni

Campylobacter jejuni infection is one of the most common enteric infections in the United States and around the world. The most common type in the United States is type 2. *Campylobacter* is microaerophilic, and fermentation in liquid media has been difficult. We studied various media and fermentation conditions and observed moderate growth in liquid media. Chemical analysis showed that the surface polysaccharides contain keto groups but no colominic acid. From its size and staining properties, we concluded that the polysaccharide is an oligo LPS.

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HUMAN IMMUNE RESPONSE TO POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES

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Surface polysaccharides of pathogenic bacteria, including capsular polysaccharides (CPS) or lipopolysaccharides (LPS), serve as both essential virulence factors and protective antigens. The age-related and T cell–independent immunogenicity of CPSs limit their use as vaccines, especially in infants and young children. LPSs are too toxic to be administered. Accordingly, their O-specific polysaccharide (O-SP), which shares the virulence-promoting and protectiveness properties of CPS, must be purified. However, O-SPS are too small to be immunogenic (haptens). Covalent binding of CPS or of O-SPS to medically useful proteins to form conjugates both increases their immunogenicity and confers T-cell dependence on these saccharides. Part of the work of this laboratory focuses on making synthetic vaccines that improve immunogenicity.

Vaccines against shigellosis

Chu, Ekborg, Sampath-Kumar, Nelson, Pozsgay, Robbins, Schneerson; in collaboration with Ashkenazi, Liptak, Passwell, Shiloach

Shigella dysenteriae type 1 and *Shigella sonnei* are Gram-negative human pathogens that cause endemic and epidemic dysentery throughout the world. Despite their discovery many years ago, there are still no licensed vaccines against these pathogens, which have developed resistance to most available antibiotics. Our approach to vaccine development against these bacteria is based on the demonstration that serum antibodies against the O-specific polysaccharides (O-SP) of Gram-negative bacteria are important for host protection. The O-SPS of *Shigella sonnei* and *Shigella flexneri* 2a were bound to bacterial toxoids. In adult army recruits and then in four- to seven-year-old children, both conjugates were safe and induced statistically significant and long-lived rises in IgG antibody levels against the homologous LPS. The conjugates induced similar though lesser increases in IgM and IgA anti–LPS levels. Re-injection of *S. flexneri* 2a conjugate induced a booster response in the recruits and the four- to seven-year-old children. A phase III trial showed that one injection of *S. sonnei* O-SP, bound to a nontoxic recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA), protected army recruits against outbreaks of this pathogen. Importantly, there was a statistically significant correlation between the levels of serum IgG anti–LPS and the efficacy of the conjugate. We developed two methods that increased the immunogenicity of the Shigella conjugates in mice: another carrier protein, a genetically inactivated *Corynebacterium diphtheriae* toxin (CRM9), was found to be a superior carrier for *S. sonnei* O-SP, and treatment of rEPA with succinic anhydride, a nontoxic mild akylating agent that converts amino groups of proteins to carboxyls, increased the immunogenicity of *S. flexneri* 2a O-SP. A phase I study in adults of these Shigella conjugates confirmed their safety and immunogenicity, although the improved immunogenicity was less marked than in mice. A Phase II study in one- to four-year-old children showed an improved immunogenicity of the new *S. flexneri* 2a conjugate but lesser immunogenicity of the *S. sonnei* conjugate. A Phase III study of the modified *S. flexneri* 2a and the original *S. sonnei* conjugates is in preparation. In collaboration with the Lanzhou Vaccine Institute and Provincial Medical Center in Henan, China, a clinical trial of the two conjugates is in the planning stage.

While O-SPs are nonimmunogenic, presumably because of their low molecular weight, covalent conjugates of an immunogenic protein and the O-SP of *S. dysenteriae* type 1 and *S. sonnei* have been shown to elicit significant anti–O-SP antibody levels of the IgG isotype, which may be boosted by repeated injections. We surmised that an improved vaccine might be constructed from chemically defined oligosaccharide fragments of O-SPs, which are devoid of biological contamination, carry a uniform molecular weight, and can be characterized by physicochemical methods.

In earlier studies, we found that, in mice, oligosaccharides as small as an octasaccharide can generate substantial amounts of O-SP–specific antibodies against *S. dysenteriae* type 1 when coupled to a protein, even if administered without an adjuvant. Based on this finding, we have directed most of our efforts in the past year to the chemical synthesis of octa-, dodeca-, and hexadecasaccharides under clean-laboratory conditions in sufficient quantities. Our earlier synthetic experience provided the foundation for this work. However, we encountered several chemical problems during scale-up that necessitated further synthetic explorations. The need for stereoselective construction of the alpha-glucosamidine linkage caused a particular difficulty. In small-scale syntheses conducted earlier, we circumvented the problem by repeated chromatographic purifications of the product mixture. On a large scale, such purifications would be laborious and prohibitively expensive. Based on the assumption that a rigid as opposed to a flexible glucosamine donor might lead to higher proportions of the alpha interglycosidic linkage, we prepared several 4,6-O-acetal–protected 2-azido-glucose derivatives and tested their reactions with a rhamnose acceptor. We found that the benzophenone acetal–protecting group in an azido-glucose donor moiety provided high stereoselectivity, presumably by cooperative factors, including rigidity of the hexopyranose ring and steric crowding on the beta side. Using this approach, we were able to achieve more than 95 percent stereoselectivity in the construction of alpha-glucosamidine interglycosidic linkages. The synthetic improvements allowed us to scale up the synthetic operations. As a result, we now have the targeted spacer-equipped synthetic polysaccharides in quantities of 300 to 400 mg. We will conjugate the preparations to a medically acceptable protein for evaluating their immunogenicities in humans.

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Pozsgay V, Vieira NE, Yergey A. A method for bioconjugation of carbohydrates using Diels-Alder cycloaddition. *Org Lett* 2002;4:3191-3194.

Recombinant vaccine against *Bacillus anthracis*

Kubler-Kielb, Majadly, Schneerson; in collaboration with Leppla, Ramirez, Shiloach, Yergey

Bacillus anthracis, a potential cause of lethal human infection, has two essential virulence factors, anthrax toxin and a capsule, without either of which it is not pathogenic for humans. The toxin is composed of three peptides that are nontoxic when administered alone: Lethal Factor (LF), Edema Factor (EF), and Protective Antigen (PA). PA is the toxin part that binds to mammalian cells. It has to have a 20 KDa peptide hydrolyzed off, exposing a site to which LF or EF may bind and rendering toxins that enzymatically modify substrates in mammalian cell cytosol. The capsule is composed of poly-(gamma-D-glutamic acid) (PGA). It is nonimmunogenic and its protective effect is unknown. The licensed vaccine is safe and protective but has limitations that justify development of improved vaccines. We isolated a recombinant PA from an unencapsulated strain grown in a fermenter and found that several formulations with formaldehyde-treated and alum-adsorbed materials were immunogenic in mice. Clinical lots are in preparation. We isolated the capsule from a nontoxic strain; the capsule or corresponding synthetic peptides were bound to BSA, rEPA, or rPA. To identify the optimal construct, we conjugated peptides of varying lengths (5-, 10-, and 20-mers) of the D or L configuration with active groups at the C- or N-termini. The conjugates were characterized by physicochemical and immunological assays and immunogenicity in five- to six-week-old mice. In contrast to PGA alone, all conjugates were immunogenic and the D-PGA-induced antibodies were opsonophagocytic, with rPA the more effective carrier.

Leppla SH, Robbins JB, Schneerson R, Shiloach J. Development of an improved vaccine for anthrax. *J Clin Invest* 2002;110:141-144.

Schneerson R, Kubler-Kielb J, Liu TY, Dai ZD, Leppla SH, Yergey A, Backlund P, Shiloach J, Majadly F, Robbins JB. Poly(gamma-D-glutamic acid) protein conjugates induce IgG antibodies in mice to the capsule of Bacillus anthracis: a potential addition to the anthrax vaccine. *Proc Natl Acad Sci USA* 2003;100: 8945-8950.

Toward a vaccine against Lyme disease

Ben-Menachem, Coxon, Kubler-Kielb, Pozsgay, Schneerson; in collaboration with Yergey

Borrelia burgdorferi, a spirochete transmitted though the bite of infected *Ixodes* ticks, is the etiologic agent of Lyme disease. The available protein vaccine is not effective under the age of 12 years. Lipopolysaccharide (LPS) has been described in other spirochetes, but its presence in *B. burgdorferi* has been debated. So far, we have not been able to confirm its presence. The search for LPS revealed two unique glycolipids: cholesteryl 6-O-palmitoyl-beta-D-galactopyranoside (BBGL1) and 1-O-palmitoyl-2-O-oleyl-3-O-alpha-D galactopyranosyl-sn-glycerol (BBGL2). Evidence suggests that the two glycolipids are surface-exposed. Injected into mice in various formulations (with CFA, PBS, DMSO, or squalene), BBGL1 induced specific antibodies, of which the combination with CFA proved most effective. The biological effect of the antibodies is under investigation.

Based on the assumption that antibodies to these glycolipids, assumed to be functional equivalents of a lipopolysaccharide, might offer protection against *B. burgdorferi*, we initiated a project to produce the glycolipids and their protein conjugates in quantities and purity suitable for immunization experiments with the synthetic antigens. We have so far synthesized the native glycolipids and their structural congeners, including the 2-O and 4-O-palmitoyl derivatives as well as the parent compound cholesteryl b-D-galactopyranoside. All the glycolipids reacted with antisera raised against the native glycolipids; the weakest reaction occurred with the unnatural isomers. We assume that conjugation to a protein would be most suitable through the methyl terminus of the palmitoyl moiety. To demonstrate that chemical functionalization at this site will not abolish antigenicity, we synthesized the 16-hydroxy-palmitoyl derivative. The reaction of this material with the antiserum demonstrated that chemical changes at the terminal position do not abolish antigenicity. Based on this finding, we are currently synthesizing a spacer-equipped derivative of the native material for chemical attachment to protein that will be used in immunization experiments.

Ben-Menachem G, Kubler-Kielb J, Coxon B, Yergey A, Schneerson R. A newly discovered cholesteryl galactoside from Borrelia burgdorferi. *Proc Natl Acad Sci USA* 2003;100:7913-7918.

Vaccines against group A *Neisseria meningitidis*

Berkin, Coxon, Yergey, Pozsgay, Robbins, Schneerson

Neisseria meningitidis group A causes endemic and epidemic meningitis, notably in the meningitis belt of Africa. Although a vaccine containing the purified capsular polysaccharide (CPS) of the bacterium has been available for years, it is not sufficiently immunogenic in infants, who are at greatest risk. Our program to develop a more efficient vaccine is based on the assumption that protein conjugates of relatively short fragments of the CPS (i.e., which can be made available by controlled chemical synthesis) of Group A *N. meningitidis* will be more immunogenic in infants than the currently available vaccine. The CPS of Group A *N. meningitidis* consists of alpha-linked N-acetyl-mannosaminyl residues that are interconnected by an anomerically located phosphodiester linkage. To improve further its immunogenicity, we conjugated the CPS to BSA by using ADH as a linker bound first to the CPS or the BSA. In contrast to the CPS alone, the conjugates were immunogenic in mice, with booster responses after second and third injections and bactericidal activity related to IgG anti–CPS levels. Conjugates of the cross-reactive polysaccharides *E. coli* K93 and *B. pumilus* SH17 did not induce anti–meningitis A CPS.

The native polysaccharide is nonstoichiometrically O-acetylated at the O-3 position. In our first approach to studying the requirements of synthetic oligosaccharides related to this polymer, we targeted the synthesis of non–O-acetylated congeners. We have designed a route to the mannosaminyl H-phosphonate moiety. Iterative condensation of the unit allowed the synthesis of a dimer and trimer of the polysaccharide's repeating unit in bioconjugatable form. We also synthesized the mannosaminyl spacer and its phosphate ester. Covalent attachment of the synthetic subunits to human serum albumin using our conjugation technology afforded glycoconjugates containing up to 30 saccharide chains per molecule of BSA. The antigenicity of the conjugates was demonstrated by the precipitin formation with the serum of a horse that was immunized with formalin-killed group A *N. meningitidis*. The experiments showed that the serum recognizes as small a component of the polysaccharide as a monosaccharide. They also showed that formation of precipitation indicates that the presence of O-acetyl groups in the polymer is not essential for antigenicity. Our current work focuses on the synthesis of higher oligomers with and without O-acetyl groups for evaluation of their immunogenicies.

Berkin A, Coxon B, Pozsgay V. Towards a synthetic glycoconjugate vaccine against Neisseria meningitidis A. *Chemistry* 2002;8:4424-4433.

Use of cross-reacting antigens to boost immungenicity

To determine if concurrent administration of a cross-reacting and a homologous CPS has an advantage over the use of the homologous CPS alone, we used conventional methods to isolate the cell wall polysaccharide (PS) of *Bacillus pumilus*, SH18, which has been reported to cross-react with the CPS of *Haemophilus influenzae* type b (HIb). We then investigated its structure by using gel chromatography–mass spectrometry, NMR, fast atom bombardment, and several sugar-degrading techniques. It was shown to be composed of polyribitolphosphate, polyribitolphosphate substituted at C2 with N-acetyl glucosamine, and polyglycerolphosphate. The CPS cross-reacted with anti–*Staphilococcus epidermidis* as well as with the anti–HIb. We conjugated the polysaccharide to carrier proteins and evaluated the conjugate's immunogenicity in GP mice. Conjugate-induced antibodies reacted with the homologous and several cross-reacting polysaccharides.

Conjugate vaccine against pneumococcal pneumonia

Schneerson; in collaboration with Jonsdottir

We conjugated the polysaccharide of *Streptococcus pneumoniae* type 6B, one of the most common and least immunogenic streptococcus types, to tetanus toxoid and then evaluated the conjugate in patients with chronic obstructive pulmonary disease as compared with the 23-valent pneumocoocal polysaccharide vaccine in these same patients and with the response of healthy young adults. We found that the antibody levels were similar in the three groups. Opsonophagocytosis was related to IgG anti–type 6B levels, suggesting that conjugate vaccines would confer protective immunity in elderly chronic obstructive pulmonary disease patients.

Jonsson S, Vidarsson G, Valdimarsson H, Schiffman G, Schneerson R, Jonsdottir I. Vaccination of COPD patients with a pneumococcus type 6B tetanus toxoid conjugate vaccine. *Eur Respir J* 2002;20:813-818.

Plasmodium falciparum

In an attempt to increase immunogenicity, we are applying techniques used in our preparation of our synthetic *S. dysenteriae* type 1 and *B. anthracis* experimental vaccines to proteins of the zygote of *P. falciparum*.

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DEVELOPMENTAL REGULATION OF NEURONAL AND MUSCLE PLASTICITY

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The general interest of the laboratory is to understand the molecular mechanisms that regulate neuronal and muscle plasticity during development and in response to experience. We are investigating how neuregulins, a family of neural factors related to EGF, regulate synaptic transmission at interneuronal synapses. These factors and their receptors have recently been implicated in the neurological disorder schizophrenia. In a second project, we are studying how different muscle types emerge during development and how they are differentially regulated by distinct frequencies of muscle depolarization.

Neuregulin effects on synaptic plasticity: differential expression in the developing central nervous system

Longart, Liu, Karavanova, Vasudevan, Chatani-Hinze, Yun, Buonanno; in collaboration with Carroll

Neuregulins (NRG 1-3) are growth/differentiation factors that signal via a family of receptor tyrosine kinases known as ErbB 1-4. Earlier work by our group and others showed that prolonged exposure of neurons to NRG-1 elicits changes in the composition of neurotransmitter receptors for glutamate (NMDA subtype), GABA, and acetylcholine. Initially, we found that the coactivation of ErbB receptors by NRG-1 and of NMDA receptors by glutamate is necessary to modify the expression of an NMDA receptor subunit gene, suggesting cross-talk between these two types of signaling pathways (Ozaki *et al.*, *Nature* 1997;390:691-694). The subsequent demonstration that ErbB4 and NMDA receptors colocalize at glutamatergic synapses with PSD-95, a PDZ protein that couples postsynaptic receptors to signaling complexes, led us to hypothesize that the NRG/ErbB signaling pathway may acutely modify synaptic properties (Garcia *et al.*, *PNAS* 2000;97:3596-3601). Recent research from our group and others supports the view that NRG-1 alters synaptic transmission. The implications of these findings for basic and clinical science may be extremely important in light of recent studies associating NRG-1 mutations with schizophrenia in Icelandic and Scottish families. Mutant mice with decreased levels of NRG-1 and ErbB receptors have a reduction in NMDA receptors and manifest behavioral deficits reported to be consistent with schizophrenia that are reversed with pharmacological agents used to treat this disorder. Future studies on the cellular and molecular targets of the NRG/ErbB signaling pathway should contribute to understanding distinct mechanisms that regulate synaptic plasticity and that may be associated with neurological disorders.

As a first step to understanding how NRGs contribute to distinct aspects of neural development and function, we characterized their regional and subcellular expression patterns in developing brain. NRG-1 expression is highest at birth while NRG-2 mRNA levels increase with development; expression of both genes is restricted to distinct brain regions. In contrast, NRG-3 transcripts are abundant in most brain regions throughout development. The marked differences in the regional and temporal expression patterns of NRGs are consistent with the idea that these factors serve different functions during development.

We generated NRG-2 antibodies to analyze the processing, expression, and subcellular distribution of this factor in central neurons. Like NRG-1, the transmembrane NRG-2 pro-protein is proteolytically processed in transfected cells and in neural tissues, and its active extracellular domain accumulates on the neuron surface. However, despite the structural similarities between NRG-1 and NRG-2, we found that both factors are targeted to distinct subcellular compartments. NRG-2 accumulates in proximal primary dendrites of hippocampal neurons in culture and *in vivo* but is not detectable in axons or synaptic terminals. We observed a similar dendritic distribution in cortical neurons and cerebellar Purkinje cells. In contrast, NRG-1 is highly expressed in axons of dissociated hippocampal neurons as well as in somas and dendrites. The distinct temporal, regional, and subcellular expression of NRGs suggests their unique and nonredundant roles in neural function. To test this hypothesis, our laboratory has begun to analyze the phenotypes of different NRG knock-out mice. By using multidisciplinary approaches, which include electrophysiology, genomics, and cellular and molecular biology, we aim to elucidate how this signaling pathway regulates synaptic plasticity. Our work should lead to a better understanding of the role of NRGs in neurological disorders.

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Developmental and activity-dependent regulation of muscle types: characterization of General Transcription Factor 3, a transcription factor that contributes to the slow muscle program

Vullhorst, Karavanova, Buonanno

The developmental and neuronal regulation of skeletal muscle fiber types provides an excellent model in which to study how patterned activity regulates plasticity of postsynaptic targets. Our long-term objectives are to identify the DNA regulatory elements and transcription factors that regulate the emergence of slow- and fast-twitch fibers during development and that are necessary to intraconvert the contractile properties of muscles in response to specific patterns of motoneuron activity. The troponin I slow (*TnIs*) and fast (*TnIf*) genes have served as our experimental model because expression of both genes is specific to fiber type and regulated by selective patterns of electrical impulses that mimic motoneuron activity.

Our earlier work showed that General Transcription Factor 3 (GTF3) is highly expressed in most tissues during early fetal development (when muscle types emerge), binds to a DNA element in the *TnIs* enhancer required for fiber-type specificity, and regulates the slow muscle program in regenerating adult muscles (Calvo *et al.*, 2001). Using GTF3-mutant mice, we are currently analyzing the role of this factor during early muscle development.

Biochemical characterization of GTF3 indicates that its reiterated HLH domains, regulated in part by differential splicing, contribute to the complexity of this nuclear factor. GTF3, like its paralog TFII-I, contains multiple HLH domains and an N-terminal leucine zipper motif. We found that HLH domain 4 is necessary and sufficient for binding to the *bicoid*-like motif (BLM) and that removal of GTF3 N-terminal sequences may unmask the DNA-binding domain. We found six GTF3 splice variants in mice. The gamma-isoform lacking exon 23 and exons 26 through 28 (encoding the sixth HLH domain) interacted most avidly with the *TnIs* DNA regulatory element while the alpha and beta isoforms that include these exons fail to bind. The GTF3 leucine zipper promotes homodimerization, but not heterodimerization with its close relative TFII-I. We speculate that a large number of GTF3 proteins with different DNA-binding properties can be generated in each cell by alternative splicing and combinatorial association of GTF3 polypeptides. The genes encoding GTF3 and TFII-I are lost in an approximately 2.0 Mb microdeletion of chromosome 7q11.2 in individuals with Williams syndrome (WS). Persons with WS have distinctive physical, cognitive, and behavioral abnormalities that include impaired spatial cognitive skills and myopathies. Although approximately 20 genes are associated with the microdeletion, recent studies strongly implicate GTF3 and TFII-I with the cognitive deficiencies observed in WS patients. Our studies using ectopically transfected GTF3 constructs in adult muscles and GTF3 knock-out mice support a possible role for this factor in regulating muscle contractile properties, which could be related to myopathies observed in WS. The observation that GTF3 and TFII-I are highly expressed in developing musculature and neurons raises the possibility that reduction of these nuclear factors during embryogenesis may affect the expression of target genes later in development.

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REGULATION OF PINEAL FUNCTION

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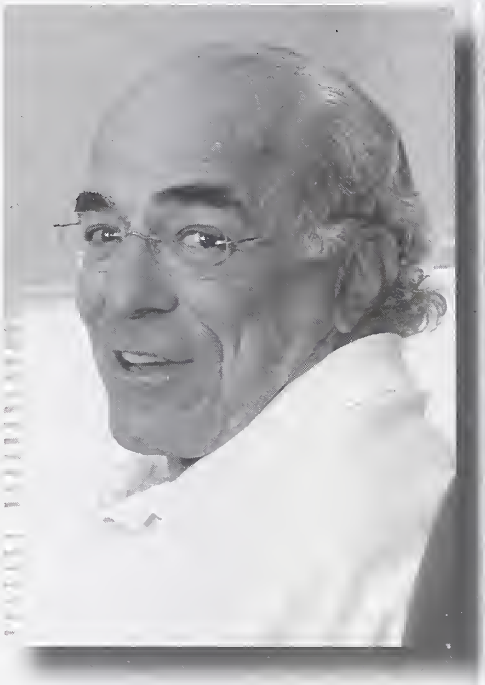

We focus on chronobiology and pineal function, investigating how production of the pineal hormone melatonin is regulated. Our research has indicated that there is broad integration of the biochemical function of the pineal gland and that, although large changes in the production of melatonin are controlled by changes in arylalkylamine N-acetyltransferase activity, there is little doubt that other factors contribute to the rhythm in melatonin. Our work has wide-ranging implications in biology and is of special interest to clinical scientists studying human diseases relating to circadian rhythms, including sleep physiology, mood disorders, and alertness.

The melatonin rhythm enzyme arylalkylamine N-acetyltransferase

Ganguly, Coon, Namboodiri, Falcón, Ho, Pollard, Weller, Klein; in collaboration with Chemineau, Cole, Dawid, Dyda, Gothilf, Hickman, Iuvone, Jaffe, Koonin, Malpaux, Schomerus, Toyama, Zhen

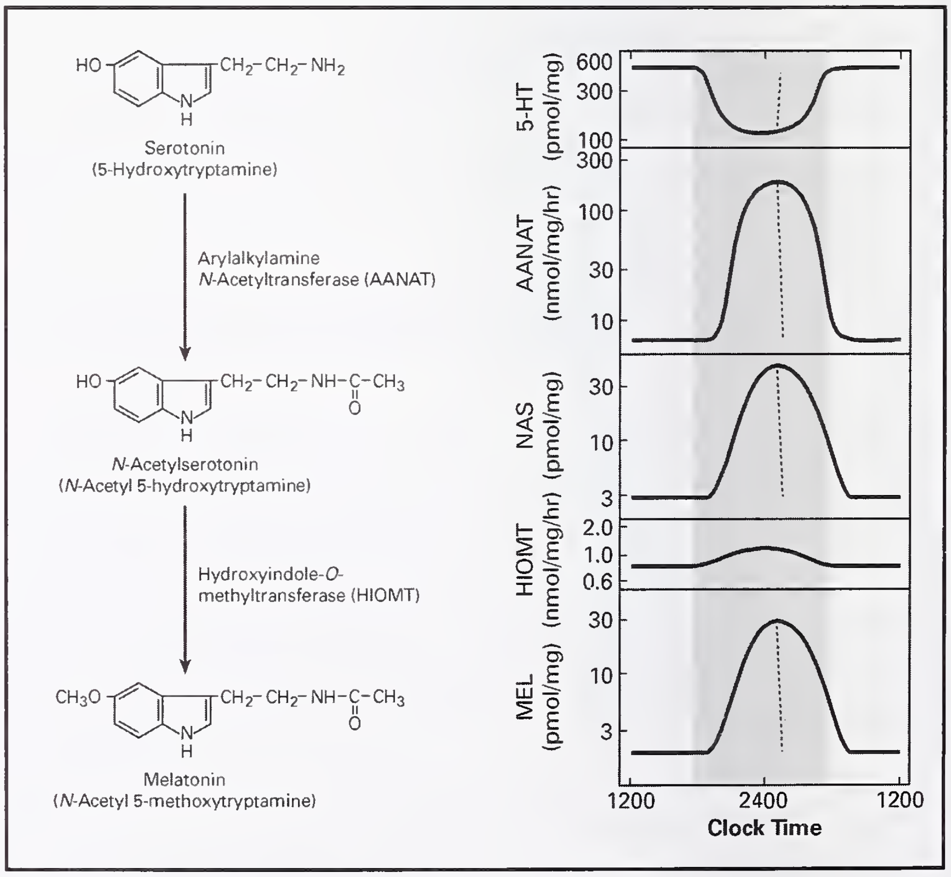

FIGURE 12.1

Schematic model of regulation of the melatonin rhythm enzyme arylalkylamine N-acetyltransferase

We have been at the forefront of investigations into arylalkylamine N-acetyltransferase (AANAT), the second enzyme in melatonin synthesis from serotonin (see Figure 12.1). A major advance in our work was the early identification of this enzyme as critical to the control of the rhythm in melatonin synthesis. In all species examined today, the large increase in melatonin synthesis at night causes an increase in the production of melatonin. Another major advance in our research was the cloning of the enzyme, which allowed us to make significant strides in understanding the transcriptional and translational control of the enzyme. Transcriptional mechanisms include interactions of cyclic AMP response elements in the AANAT promoter and other response elements in the promoter that also appear to be involved, making expression of the enzyme highly tissue-specific. These transcriptional events control both tissue specificity and, in some but not all vertebrates, the rhythmic control of expression of the AANAT gene. For example, we have determined that, in rodents, there is an approximately 100-fold increase in the expression of the AANAT gene at night, whereas ungulates and primates experience only a small increase. The results have demonstrated that transcriptional control mechanisms cannot be viewed as essential in controlling melatonin synthesis in all vertebrates; as discussed below, post-translational mechanisms play an important role.

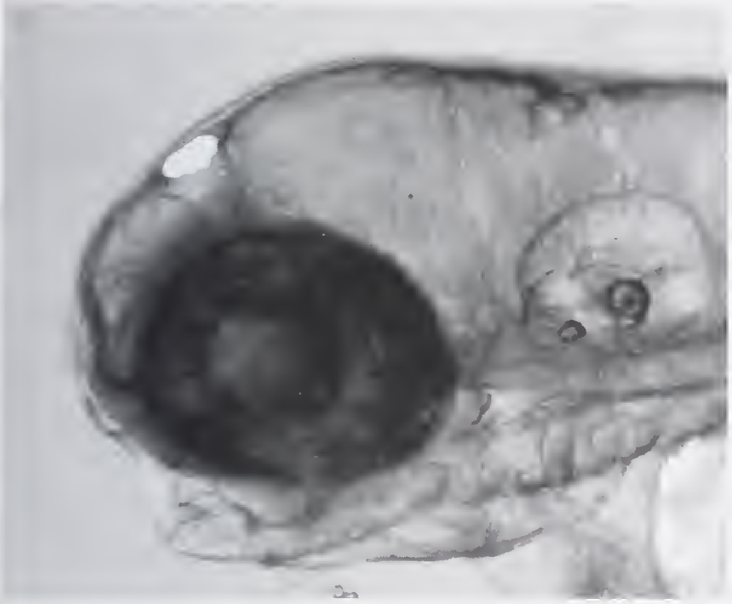

FIGURE 12.2

Expression of green fluorescent protein under control of the arylalkylamine N-acetyltansferase promoter in the zebrafish pineal gland (white patch)

A recent development in understanding the transcriptional control of AANAT has been the production, through the collaborative efforts of our staff and the Laboratory of Molecular Genetics, of a transgenic zebrafish that expresses a reporter gene under the control of the AANAT promoter (Figure 12.2). The bright area in the center of the fish head is the fluorescently labeled pineal gland. It is a model system that will allow investigators to probe the factors that control the expression of the AANAT gene during development and to determine the factors controlling pineal tissue specificity.

In a similar approach, we are collaborating with researchers at Tel Aviv University and in the Laboratory of Molecular Genetics to identify critical elements in the AANAT gene involved in regulation of tissue-specific expression. Specifically, we have injected eggs with the fluorescent marker under the control of portions of the AANAT gene in which specific sites have been altered.

In addition to describing the transcriptional factors controlling the expression of AANAT, we have made advances in understanding post-translational regulation of the enzyme. Sequence analysis has revealed that all AANATs contain C- and N-terminal sites for phosphorylation by cyclic AMP–dependent protein kinase. We have studied the sites and determined that they are critical in the control of the stability and biological half-life of the molecule (Figure 12.3). Phosphorylation of these sites controls binding of AANAT to a protective binding partner, 14-3-3 protein. Binding protects the enzyme from destruction while reversal of the binding can lead to destruction. The balance between destruction and protection is governed by the phosphorylation-dependent binding of AANAT to 14-3-3. In collaboration with NIDDK investigators, we determined the molecular basis of the interaction through analysis of the crystal structure formed by AANAT and 14-3-3- proteins. Current research has focused on one of the phosphorylation sites

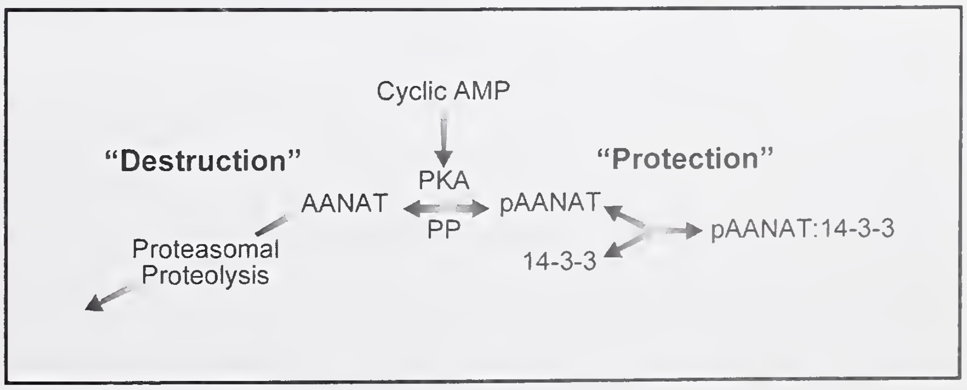

FIGURE 12.3

of AANAT, which are located in the C-terminal region. The work of Joan Weller has resulted in the development of highly specific antisera, which she has used to determine the phosphorylation state of the enzyme. Her work has demonstrated that the C-terminal site is phosphorylated at night and that the phosphorylation state of the enzyme decreases immediately after animals are exposed to light, probably leading to destruction of the enzyme.

Surajit Ganguly has explored the functional role of phosphorylation of AANAT in two ways. First, he has established a highly sensitive method for measuring the binding of AANAT to 14-3-3. The method has underscored the importance of phosphorylation and points to the significance of the entire AANAT molecule, which appears to contribute considerably to the binding affinity toward 14-3-3 protein. Using his system, Ganguly will study the relative importance of the C- and N-terminal PKA sites. He has also been involved in analysis of the role played by phosphorylation and 14-3-3 protein in controlling the kinetics of acetylation.

In a collaborative effort with Phil Cole, we have used novel synthetic approaches to examine the role of phosphorylation in controlling AANAT and have formulated AANATs that contain nonhydrolyzable phosphate at the N-terminal PKA site. We have injected the AANATs into cells and then monitored their biological half life by using antisera. The results clearly indicate that the nonhydrolyzable phosphate at the N-terminal PKA site prevents destruction of AANAT. The importance of such post-translational regulatory mechanisms is apparent from studies on the monkey pineal AANAT performed by Steven Coon. The studies have established that large changes occur in the abundance of AANAT protein and activity and that AANAT mRNA, i.e., gene expression, does not change. Accordingly, it appears that the primary mechanism for regulation of AANAT protein in primates is post-translational.

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Global analysis of gene expression

Coon, Gaildrat, Ganguly, Morin, Kim, Weller, Klein; in collaboration with Baler, Blackshaw, Carter, Hogenesch, Humphries, Møller, Munson

We have initiated several projects aimed at obtaining a global picture of differences in gene expression that occur on a night/day basis and in identifying genes that are highly enriched in the pineal gland. We initiated our work with resources that provided an opportunity to analyze a relatively small number of genes and have expanded into the use of microarrys, which allow investigators to examine tens of thousands of genes and ESTs. In addition to using commercially available microarrays, we have participated in the NICHD initiative to develop a zebrafish microarray, which became commercially available in 2003. Our efforts toward understanding pineal gene expression have involved several species and have already identified a set of genes highly expressed in the pineal gland. Steven Coon is coordinating the work, which involves a collaborative effort with Peter Munson at CIT.

The group of genes expressed specifically in the pineal gland of several vertebrates includes the well-known genes associated with melatonin production and visual signal transduction. In addition, we have identified a number of genes that are new to the pineal literature, leading to a rapid increase in our knowledge of the biochemical profile of the pineal gland conserved across species. The genes are also pointing to new transcriptional pathways controlled by previously unrecognized transcription factors; through the analysis of these transcription factors and the promoters of genes that are either up-regulated at night or highly expressed in the pineal gland, we will be able to construct a regulatory network that describes the cascade of transcription factors underlying the control of pineal gene expression. Beyond obtaining a more complete understanding of gene expression, our work has led to the identification of new and potentially important pathways involved in cell-cell communication and signal transduction in the pineal gland. Moreover, expression of some genes suggests new functions for the pineal gland.

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Regulation of S-adenosylmethionine synthesis

Kim, Klein; in collaboration with Charlton

S-adenosylmethionine is the cofactor of the last enzyme in melatonin synthesis. Results from our gene-profiling efforts have highlighted the synthesis of this cofactor. Work by Jong-So Kim has determined that the expression the enzyme methionine adenosyl transferase 2a, which catalyzes the production of this cofactor in the pineal gland, increases at night as does the enzyme's activity and concentration. The molecular basis of the increase involves neural stimulation of the pineal gland by norepinephrine, which results in the elevation of cyclic AMP. The increase in S-adenosylmethionine (SAM) synthesis at night is obviously linked to the increased requirement for this methyl donor. Regulation of the synthesis of SAM by neural mechanisms has not been previously described. However, the cofactor plays a central role in the synthesis and metabolism of many transmitters (catecholamines, indoles, histamine, and so forth). Accordingly, evidence from the pineal gland that the activity of MAT 2a can be regulated by a neural circuit via a cyclic AMP mechanism points to the possibility that activity of MAT 2a might be regulated by transmitters in other brain regions and that the levels of SAM might be controlled through pharmacological manipulation of MAT 2a expression.

Regulation of acetyl coenzyme A synthesis

Morin, Klein; in collaboration with Benjamin

Fabrice Morin has studied ATP-dependent citrate lyase, the enzyme that regulates the formation of acetyl coenzyme A. His work has established that the pineal expresses very high levels of the enzyme compared with other tissues and that, in some species, the abundance of mRNA encoding the enzyme exhibits daily changes. Morin has obtained evidence of a physical interaction between ATP-dependent citrate lyase and AANAT, potentially bringing the source of acetyl coenzyme A directly into contact with AANAT and promoting efficient acetylation of serotonin and melatonin production. It appears possible that this association might be regulated by phosphorylation and that it could provide an important element in the general activation of the pineal gland supporting the nocturnal increase in melatonin production.

Induction of membrane protein

Gaildrat, Klein; in collaboration with Ganapathy, Inui

Pascaline Gaildrat has determined that one of the features of nocturnal activation of the pineal gland involves the expression of a gene that encodes a membrane protein involved in transfer of material across the membrane. Her work has revealed large changes in both the mRNA encoding the protein and the protein itself, representing a unique mechanism of regulation not typically seen with membrane proteins, which are usually relatively stable and do not appear and disappear rapidly. She has also discovered that the expression of the gene product is highly restricted to the pineal gland and has identified a section of the gene that appears to be responsible for this pattern of tissue distribution and for the night/day pattern of expression.

Metal biology

Klein, Shi, Gaildrat, Ganguly

Enzymes involved in indole synthesis and metabolism require metals. The concentration of divalent ions including copper and zinc are governed by metallothionines, small proteins dedicated to binding and buffering these ions. Qiong Shi has discovered that expression of one form of these proteins is governed by an adrenergic cyclic AMP mechanism the pineal gland. He has determined that both the protein and mRNA encoding the enzyme are under the control of the second messenger.

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SECRETORY PROTEIN TRAFFICKING AND GRANULE BIOGENESIS IN NEUROENDOCRINE CELLS

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Yazmin Rodriguez-David, BS, *Postbaccalaureate Fellow*
Chunfa Zhang, PhD, *Guest Researcher*

We study the cell biology of endocrine and neuroendocrine cells. Our focus is two-fold: to investigate the mechanisms of biosynthesis and intracellular trafficking of peptide hormones and neuropeptides and their processing enzymes and to uncover mechanisms involved in the regulation of dense-core secretory granule biogenesis. Our work has led to the discovery of novel molecular mechanisms of protein trafficking to the regulated secretory pathway and has identified players that control secretory granule biogenesis. Studies using cell lines, primary cell cultures, and mouse models have provided a better understanding of diseases related to defects in hormone targeting and cholesterol deficiency.

Mechanism of sorting pro-neuropeptides and neurotrophins to the regulated secretory pathway

Cawley, Zhang, Rodriguez-David, Lou, Loh

The intracellular sorting of pro-neuropeptides and neurotrophins to the regulated secretory pathway (RSP) is essential for the processing, storage, and release of active proteins and peptides in the neuroendocrine cell. We investigated the sorting of pro-opiomelanocortin (POMC, pro-ACTH/endorphin), pro-enkephalin (pro-ENK), and brain-derived neurotrophic factor (BDNF) to the RSP. We showed that, as a concentration step, these pro-proteins undergo homotypic oligomerization as they traverse the cell from the site of synthesis in the endoplasmic reticulum to the trans-Golgi network (TGN), where they are sorted into the dense-core granules of the regulated secretory pathway for processing and secretion. Site-directed mutagenesis studies identified a consensus sorting motif consisting of two acidic residues, 12 to 15Å apart and exposed on the surface of these molecules, and two hydrophobic residues, 5 to 7Å away from the acidic residues that are necessary for sorting to the RSP. We identified the RSP sorting receptor membrane carboxypeptidase E (CPE) that was specific for the sorting signals of POMC, pro-enkephalin, and BDNF. The two acidic residues in the prohormone/pro-BDNF sorting motif interact specifically with two basic residues, R255 and K260, of the sorting receptor carboxypeptidase E (CPE) to effect sorting to the RSP. Transfection of a mutant CPE with R255 and K260 mutated to A in a CPE null clone of Neuro2a cells and transfection of a dominant negative CPE mutant into AtT-20 cells caused mis-sorting of POMC to the constitutive pathway, indicating that, *in vivo,* the basic residues in the sorting domain of CPE interact with the acidic residues in the POMC sorting signal to effect sorting to the RSP.

Using a mouse model synthesizing a mutant CPE that is degraded in the pituitary, we were able to show mis-sorting of endogenous POMC in these cells. The studies provide evidence for a sorting signal/receptor–mediated mechanism for targeting prohormones to the regulated secretory pathway in neuroendocrine cells.

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Sorting of normal and proinsulin mutants in hyperproinsulinemia patients

Dhanvantari, Zhang, Loh; in collaboration with Mackin, Morris

Investigations into the sorting of proinsulin in pancreatic beta-cells has identified an RSP sorting signal in proinsulin similar to POMC. In monomeric proinsulin, the sorting signal motif consists of residues E13 and L17 located on the B chain and L16 and E17 located on the A chain. In hexameric proinsulin, residue E13 on the B chain is buried, and the motif consists of the two residues in the A chain from two adjacent proinsulin dimers in the hexamer. Depletion of CPE in these cells using siRNA and the use of a dominant negative mutant of CPE demonstrated that CPE acts a sorting receptor for proinsulin in beta-cells.

To understand the molecular basis of hyperproinsulinemia, we investigated the intracellular sorting of genetically mutated proinsulins found in patients with abnormally high levels of plasma proinsulin. We discovered that one form of mutant proinsulin found in these patients, HisB10Asp, which is unable to hexamerize but forms dimers, was mis-sorted to the constitutive pathway and secreted in an unregulated manner when transfected into a cell line. Molecular modeling of the dimer of this mutant proinsulin predicted that the molecular distance of the two acidic residues of the RSP sorting signal motif would be too large to allow interaction with the basic residues in the binding site of the sorting receptor CPE. Indeed, *in vitro* binding studies showed that the mutant did not bind to CPE, resulting in its inability to be sorted to the RSP for processing to insulin and secretion in a secretagog-dependent manner. Arg65Pro and Arg65Leu hyperproinsulinemia proinsulin mutants were also found to be secreted constitutively and not stored. Binding studies showed that mutant Arg65Pro and Arg65Leu proinsulins bind poorly to CPE, accounting for the lack of sorting and retention in the immature secretory granules. The high levels of secreted mutant proinsulins in the plasma of hyperproinsulinemia patients are therefore attributable to defects in the sorting of these molecules as a consequence of their genetic structural alterations.

Dhanvantari S, Shen FS, Adams T, Snell CR, Zhang C, Mackin RB, Morris SJ, Loh YP. Disruption of a receptor-mediated mechanism for intracellular sorting of proinsulin in familial hyperproinsulinemia. *Mol Endocrinology* 2003;17:1856-1867.

Phenotypic characterization of the CPE knockout mouse

Cawley, Loh; in collaboration with Hill, Wetsel

To test the function of CPE as a sorting receptor *in vivo*, we generated a CPE knock-out (KO) mouse by deleting exons 4 and 5 from the CPE gene and then characterized its phenotype. Behavioral analysis of the CPE KO mice carried out by Joanna Hill showed that the KO mice are docile, with diminished aggressiveness and sensitivity to pain. KO pups are runts for the first two weeks of age but become obese by 10 to 12 weeks of age. The obesity continues to develop until the mice reach weights of over 70g for both males and females at 40 weeks of age. In some cases, death occurs presumably as a result of obesity-related complications. To date, about 20 percent of the KO mice have died as compared with none of the control animals. Fasting levels of glucose and insulin were monitored regularly and found to be elevated in the KO mice. The onset of diabetes was evident at about 20 weeks of age and continues to be monitored in the older mice. Surprisingly, fasting levels of insulin-like immunoreactive (IR) material in the sera of the KO animals, which was subsequently identified as proinsulin, were more than 100 times higher those of normal animals, consistent with an elevated rate of basal secretion from the pancreas, as was the elevation of secreted IR from isolated pancreatic islets of KO animals. However, stimulated secretion of (pro)insulin from the KO islets was lower than from wild-type islets. The results indicate that the regulated secretory pathway in the beta-cell of KO animals is impaired, supporting a role for CPE as a sorting receptor for proinsulin *in vivo*. Furthermore, the CPE KO mouse may serve as an excellent model for obesity and neonatal nervous system development.

Role of cholesterol in prohormone processing enzyme sorting and granule biogenesis

Arnaoutova, Assadi, Lou, Gondre-Lewis, Loh; in collaboration with Birch, Porter, Sharpe, Snell

Our recent studies showed that the prohormone and neuropeptide processing enzymes CPE and prohormone convertases 1 and 2 (PC1 and PC2) are transmembrane proteins with an atypical membrane-spanning domain at the C-terminus. In neuroendocrine cells, the enzymes are sorted at the TGN into granules of the RSP by a novel mechanism involving insertion of their C-terminal transmembrane domain into cholesterol-glycosphingolipid–rich microdomains known as lipid rafts. Removal of cholesterol from secretory granule membranes resulted in the inability of CPE, the RSP sorting receptor, to bind to cargo; cholesterol depletion by treatment of cells with lovastatin resulted in the lack of sorting of CPE to the RSP. Thus, membrane association with cholesterol-rich lipid rafts is essential for sorting of the prohormone processing enzymes to the TGN.

To determine the role of cholesterol in dense-core secretory granule biogenesis and maturation *in vivo,* we used the Smith-Lemli-Opitz syndrome (SLOS) mouse model. SLOS is a developmental disorder caused by a defect of 7-dehydrocholesterol reductase (DHCR7), an enzyme necessary for the last step in cholesterol synthesis. Morphological analysis of neonatal exocrine pancreas zymogen granules by light and electron microscopy showed a marked decrease in the number of granules in SLOS versus control mice. Of the granules present in SLOS animals, most were of an immature phenotype as compared with control animals, appearing as partially formed spheres (see Figure 12.4). Thus, genetic inhibition of cholesterol synthesis in SLOS impairs RSP granule biogenesis and maturation, leading to deficits in the secretory function in the endocrine and nervous systems.

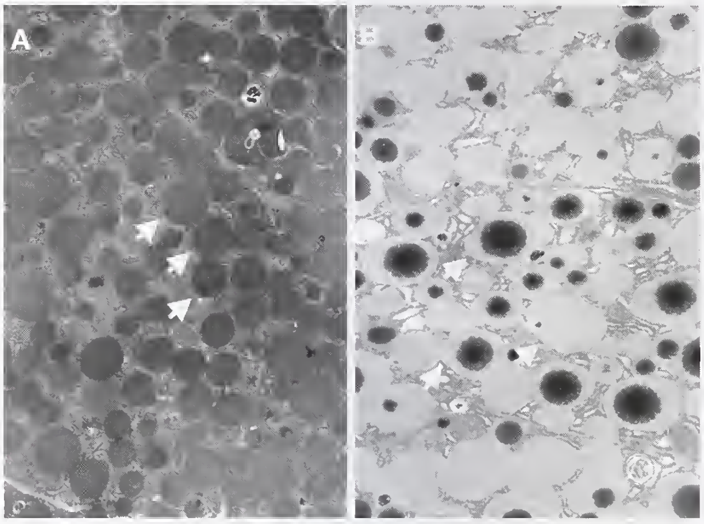

FIGURE 12.4

Electron micrographs of zymogen secretory granules from the exocrine pancreas of a normal (A) and a cholesterol-deficient SLOS-affected mouse (B)

Arnaoutova I, Smith AM, Coates LC, Sharpe JC, Dhanvantari S, Snell CR, Birch NP, Loh YP. The prohormone processing enzyme PC3 is a lipid raft-associated transmembrane protein. *Biochemistry* 2003;42: 10445-10455.

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Regulation of secretory granule biogenesis by chromogranin A

Kim, Arnaoutova, Loh; in collaboration with Cheng, Eiden

Formation of large dense-core granules (LDCG) at the TGN is essential for regulated secretion of hormones and neuropeptides from neuroendocrine cells. Our recent studies uncovered chromogranin A (CgA), an on/off switch that controls the formation of LDCG in neuroendocrine cells. Depletion of CgA in rat PC12 cells by using antisense technology resulted in the loss of LDCG and regulated secretion, and degradation of granule proteins, including CgB and synaptotagmin. Overexpression of bovine CgA in these cells rescued the wild-type phenotype. In the mutant endocrine cell line 6T3, which lacks CgA, LDCGs, and regulated hormone secretion, transfection of CgA restored the wild-type phenotype. We have recently identified the Golgi apparatus as the site of degradation of the secretory granule proteins in the absence of granule biogenesis. Thus, we propose that regulation of the stability of granule proteins at the Golgi by CgA may be a point of control of granule biogenesis in neuroendocrine cells. Supporting this hypothesis, we have recently found a protease inhibitor in the Golgi whose expression is coordinately regulated with CgA.

Recently, we used microarrays to compare gene expression in 6T3 cells lacking LDCGs and 6T3 cells stably transfected with CgA. We found that the *aquaporin-1*(AQP1, a water channel) and *granuphilin* genes, both encoding secretory granule proteins, were significantly up-regulated in 6T3 cells expressing CgA. The findings suggest that CgA may play a previously unknown regulatory role in secretory granule protein expression at the transcriptional level. From these studies, we hypothesize that CgA, either directly or indirectly, regulates secretory granule biogenesis by adjusting the secretory granule protein levels at the transcriptional and post-translational level.

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TETANUS AND BOTULINUM NEUROTOXINS AND NEURONAL CELL BIOLOGY

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Andrew G. Parfitt, PhD, *Guest Researcher*
Jennifer Kalck-Lewis, BS, *Biologist*

Using biochemical and morphologic techniques, our research focuses on synaptic function in primary neuronal cell cultures. Studies take advantage of the clostridial neurotoxins (tetanus and seven serotypes of botulinum) whose catalytic domains are zinc endopeptidases, which block vesicular neurotransmitter release by cleaving specific proteins implicated in synaptic vesicle fusion with the presynaptic membrane. Consequently, the toxins have become valuable tools for understanding neurotransmitter release, membrane trafficking, and protein sorting, transport, and targeting. The identity of toxin receptor(s), the organelles involved in toxin uptake, the mechanism of toxin translocation into the neuronal cytoplasm, intracellular trafficking of the toxins, and toxin effect on synaptic vesicle trafficking remain subjects of active research. Intoxication *in vivo* with botulinum neurotoxin causes a fatal flaccid paralysis of voluntary muscle. Because of this paralytic effect, the toxins are important therapeutic agents for a number of neurologic disorders characterized by excessive muscle contraction, including cerebral palsy. Botulinum poisoning remains a public health problem, and the toxin is considered a potential agent of bioterrorism; a more complete understanding of its intracellular functioning will aid in designing an appropriate therapy.

Uptake and translocation of botulinum neurotoxin

Keller, Neale, Kalck-Lewis

Botulinum neurotoxins act within the synaptic terminal to block neurotransmitter release. Toxin entry into the neuron is achieved by binding to neuronal membrane receptor(s), uptake into an endosome-like compartment, and penetration of the endosome membrane by a pH-dependent translocation process. Once within the synaptic cytoplasm, BoNT serotypes A and E cleave separate sites on the C-terminus of the neuronal protein SNAP-25, one of the SNARE proteins required for synaptic vesicle fusion. In the present study, we measured the effect of brief toxin exposure on SNAP-25 proteolysis in neuronal cell cultures as an indicator of toxin translocation. The results indicate that (1) uptake of both BoNT/A and BoNT/E is increased with K^+-induced depolarization in the presence of Ca^{2+} and that (2) translocation of BoNT/A from the acidic endosomal compartment is slow compared with that of BoNT/E. Polyclonal antibody against each toxin protects cells when applied with the toxin during stimulation but has no effect when added immediately after toxin exposure, indicating that toxin endocytosis occurs with synaptic activity. Both serotypes cleave from about 10 to 90 percent of SNAP-25 at concentrations between 50 pM and 4 nM. Inhibition of the vacuolar ATPase by pretreating cultures with concanamycin A (ConA) prevents cleavage of SNAP-25 by both BoNTs with IC_{50} values of about 25 nM. Addition of ConA up to 15 minutes after toxin exposure abrogates BoNT/A action; after 40 minutes, ConA is no longer protective. In contrast, ConA decreases but does not prevent translocation of BoNT/E even when added immediately after toxin exposure, indicating that BoNT/E trafficking proceeds beyond a pH-sensitive step more rapidly than BoNT/A. Our study suggests that synaptic vesicle recycling mediates uptake of BoNT/A and BoNT/E and that these two toxin serotypes are trafficked differently after uptake.

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MECHANISMS OF NEURODEVELOPMENT IN HEALTH AND DISEASE

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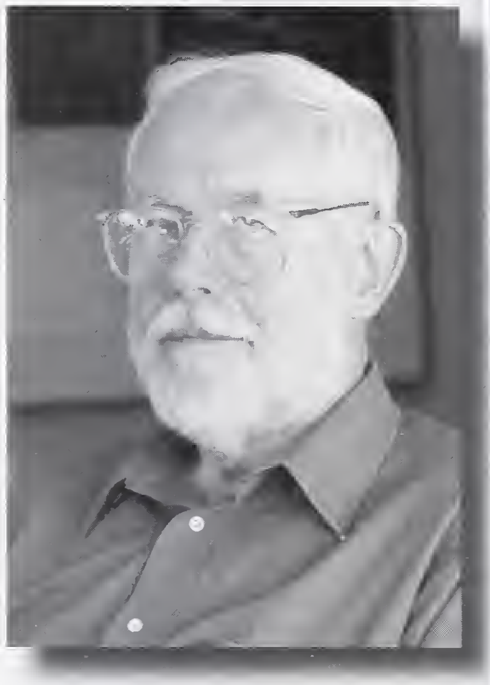

We are studying the effect of activation on synapse structure and function during development *in vitro* and *in vivo*. Activity-dependent synapse elimination is a major process determining nervous system performance, and we present evidence that different protein kinases act on receptors or other molecules at the synapse as a critical step in the synapse elimination process. We seek to extend our studies to some neurodevelopmental disorders such as autism.

Activity-dependent synapse loss and stabilization at the neuromuscular junction

P. Nelson, Jia, Li, Yang, Lanuza, Viloria, Gizaw

We have used a compartmental tissue culture system (see Figure 12.5) to analyze the cellular locus of some of the processes involved in activity-dependent synapse modulation. Two physically separate populations of cholinergic neurons (in the side compartments of the system) converge on a single population of muscle fibers. A subpopulation of the muscle fibers become innervated by axons from both of the neuronal populations. Stimulation of one set of axons produces down-regulation of the synaptic efficacy of the synapses from the other, nonstimulated neuronal population in those muscle cells innervated by both populations. Previous work showed that protein kinase C (PKC) action was required for expression of this Hebbian, activity-dependent synapse modification. The work involved pharmacological activation and blockade of PKC and the use of cells from animals in which the PKC theta isoform had been knocked out. Given that the muscle cells and neurons are prepared and plated separately, we were able to assess the effect of selective loss of PKC from neurons or muscle cells by using heterologous preparations in which either nerve or muscle lacked the PKC while the other cell type was normal.

We had evidence that the down-regulation of synapse efficacy involves postsynaptic changes, including loss of acetylcholine receptors (AChR). As we therefore expect, when a normal nerve innervates muscle lacking PKC theta, we find no activity-dependent synapse modulation. More surprisingly, the modulation is also not expressed when a PKC theta–deficient nerve innervates normal muscle. This is true with regulation induced by either electrical stimulation or PKC activation by application of the phorbol ester PMA. Furthermore, the effect of neuronal PKC theta deficiency is itself activity-dependent. In preparations lacking PKC theta in the neuronal component and that are electrically silenced with the Na^+ channel blocker tetrodotoxin, PMA does produce synapse

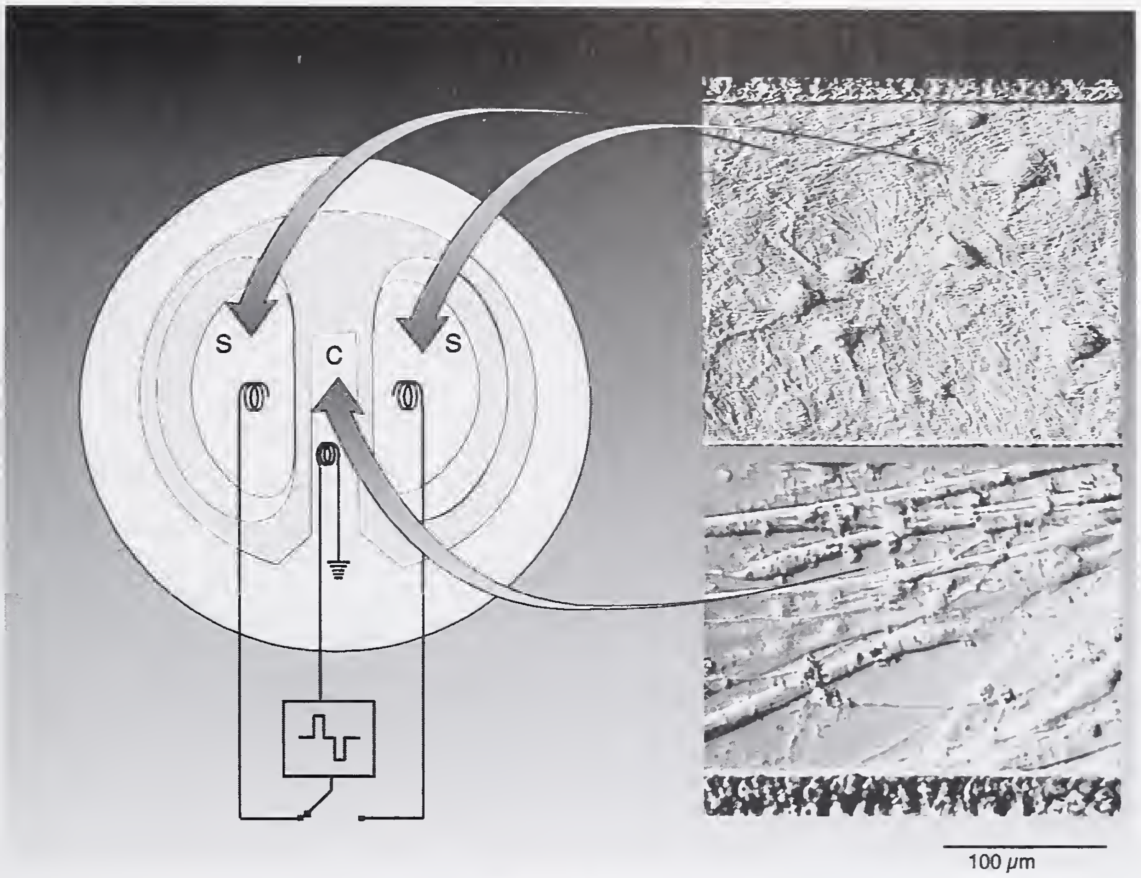

FIGURE 12.5

Figure 12.5 Legend: Diagram of Campenot chamber used in our experiments. Motoneurons from the ventral horn of embryonic mouse spinal cord were grown in side chambers (S) with the neurites extending to the central chamber beneath the barrier. Dissociated skeletal muscles from newborn mouse were cultured in the central chamber (C). Stimulation electrodes were placed in the two side chambers and current was passed beneath the barrier from one or the other side chamber to the common electrode in the central chamber.

Upper right: Ventral horn neurons growing in the side chambers following purification by metrizamide gradient centrifugation

Lower right: Dissociated muscles grown in the central chamber

down-regulation. We interpret these rather complex findings, and other findings with protein kinase A (PKA) agonists and antagonists, in terms of the model shown in Figure 2. PKA and PKC play antagonistic roles in both pre- and postsynaptic components of the synapse. PKA actions are necessary to sustain transmitter output from the nerve terminal and to stabilize the postsynaptic receptor. PKC, by contrast, blocks release of receptor-stabilizing material, perhaps a peptide (such as Calcitonin Gene–Related Peptide or CGRP) from the nerve, and acts in the muscle to destabilize the AChR. Considerable evidence from the literature suggests that this "phosphorylation" model may underlie some examples of activity-dependent synaptic plasticity in the central nervous system.

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Autocrine function for GDNF at the neuromuscular junction

Yang, P Nelson

Glia Derived Neurotrophic Factor (GDNF) is a trophin known to be produced by muscle and to have powerful effects on spinal motor neurons and other nerve cells throughout the brain. We have examined the possibility that GDNF could also have an effect on the muscle cell itself, in particular on the metabolism and membrane localization of the acetylcholine receptor (AChR). We find that GDNF does indeed increase the insertion rate of AChR into the surface membrane of muscle cells, with a lesser effect on receptor loss from the membrane and without any appreciable effect on receptor synthesis. Several inhibitor studies suggest that the GDNF effect is mediated by the alpha 1 GDNF receptor and involves the Ret receptor, MAP kinase, cAMP/CREB, and Src kinase activity. Thus, the trophin may act in a synergistic pre- and postsynaptic manner to modify synapse efficacy.

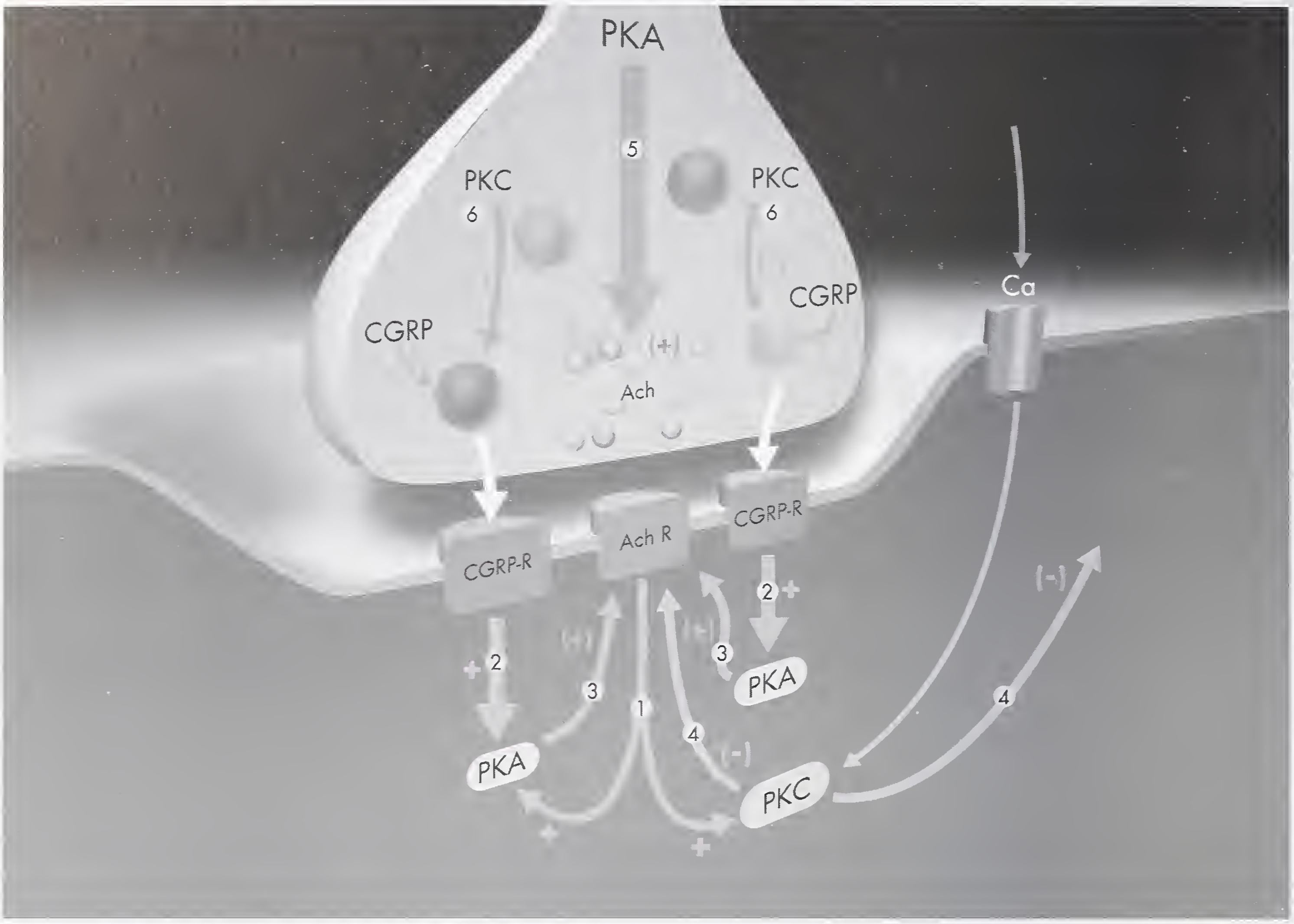

FIGURE 12.6

Diagram showing the effects of the kinases on several components of the synaptic transmission process.

1- AChR may activate both PKC and PKA
2- Peptidergic (CGRP) activation activates PKA locally
3- PKA stabilizes the AChR locally
4- PKC destabilizes AChR generally
5- Neuronal soma-derived PKA maintains transmitter output
6- Presynaptic PKC inhibits peptide (CGRP) release

Molecular basis for neurodevelopomental disorders

P. Nelson, Satyanarayana, Song, Kuddo, VanDunk; in collaboration with Grether, K. Nelson

We have access to archived neonatal blood spots from normal children and children subsequently found to be autistic. The small volumes of the available samples, the low levels of some of the analytes of interest, and the high levels of proteins, including hemoglobin, in the material eluted from the blood spots have posed considerable technical problems. We find that high levels of materials that interfere with the assay for Brain Derived Neurotrophic Factor (BDNF) are present in the blood eluates. With a combination of sample dilution with an assay buffer and the use of known analyte "spikes," we show that samples from autistic and control children do not differ either in the levels of BDNF or the levels of the interfering substances. Similarly, levels of IL-1, IL-4, IL-8, TNF alpha, and VEGF do not distinguish cases and controls in our sample.

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MITOTIC REGULATION IN HIGHER EUKARYOTES BY RAN AND SUMO-1

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We wish to understand the cell cycle checkpoints that regulate mitosis. Our studies concentrate on two closely linked biochemical pathways that have been implicated in both the regulation of mitosis and nuclear-cytoplasmic trafficking: the Ran GTPase pathway and the SUMO conjugation pathway. SUMO proteins are a conserved family of ubiquitin-like proteins that become covalently conjugated to cellular proteins in a manner similar to ubiquitin. In mammals, three SUMO isoforms all use common enzymes for their conjugation. In recent studies, we have demonstrated that the spindle checkpoint is responsive to Ran-GTP and that Ran plays a pivotal role in the metaphase-anaphase transition. We are currently investigating how, during mitotic exit, Ran-GTP contributes to activation of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase responsible for targeting key mitotic regulators for proteaosomal proteolysis. We have also shown that topoisomerase-II is a major mitotic substrate of SUMO-2 conjugation during mitosis. Inhibition of SUMO conjugation causes defects in chromatid separation. In conjunction with our earlier experiments showing that SUMO-1 conjugation regulates the Ran pathway in mitosis through RanGAP1, our observations imply that conjugation of SUMO family proteins to critical mitotic substrates may coordinate multiple events during mitotic progression.

The small ubiquitin-like modifier SUMO-1 in higher eukaryotes

Anan, Ayaydin, Azuma, Hang, Joseph, Tan, Dasso

Fission and budding yeast each contain a single SUMO family protein, and the SUMO proteins have been implicated in the regulation of the cell cycle in both organisms. In the three SUMO isoforms in mammals, the conjugation pathway for all isoforms is similar to the ubiquitin conjugation pathway: SUMO proteins must be processed to yield a C-terminal di-glycine motif. After processing, the first step in the SUMO conjugation pathway is the ATP-dependent formation of a thioester bond between SUMO proteins and their activating (E1) enzyme. The second step is the formation of a thioester bond between SUMO proteins and their conjugating (E2) enzyme, Ubc9. In the last step, an isopeptide bond is formed between SUMO proteins and substrates through the cooperative action of Ubc9 and protein ligases (E3). To understand the biological role(s) of this pathway, particularly with respect to mitosis and cell cycle progression, we are examining SUMO conjugation targets and SUMO pathway enzymes in a number of ways.

We used *Xenopus* egg extracts to examine cell cycle–dependent changes in SUMO-conjugated proteins. We found a set of high molecular weight, chromatin-dependent mitotic SUMO-containing species, which protein sequencing revealed to be SUMO-conjugated topoisomerase-II. Topoisomerase-II is modified exclusively by SUMO-2/3 during mitosis under normal circumstances; the modification is maximal in metaphase, followed by rapid deconjugation during anaphase. The differential extraction properties of modified and unmodified topoisomerase-II suggest that SUMO-2/3 conjugation may mobilize topoisomerase-II from mitotic chromatin in a manner that is important for chromosome segregation. The results of analyses using dominant negative forms of Ubc9 and topoisomerase-II inhibitors are further consistent with this idea. Together, our findings indicate that SUMO-2/3 conjugation of topoisomerase-II is important for remodeling of mitotic chromosomes at the metaphase-anaphase transition and that failure of such remodeling could be expected to cause high levels of chromosome mis-segregation *in vivo*.

Ran-GTP has an important role in regulating the organization of the cell during both interphase and mitosis (see "Mitotic roles of Ran GTPase"). Given this role, knowledge of the distribution of Ran regulators will be essential for understanding the control and function of this pathway. In metazoans, RanGAP1 is conjugated to SUMO-1. SUMO-1 modification causes RanGAP1 to associate with Ubc9 and RanBP2, a large nuclear pore protein with Ran-GTP binding domains and a SUMO E3 ligase domain. In previous studies, we examined the behavior of RanGAP1 during mitosis and found that RanGAP1 associates with kinetochores through a SUMO-1–dependent mechanism. RanBP2 co-localized with RanGAP1 on spindles, suggesting that a complex between these two proteins may be involved in mitotic targeting of RanGAP1. Recently, we have examined the structural requirements for targeting RanGAP1 and RanBP2 as well as their function in mitosis. We found that the RNAi against RanBP2 displaced RanGAP1 from kinetochores, supporting the notion that these proteins target to kinetochores as part of a single complex. Both proteins were displaced after RNAi-mediated depletion of integral kinetochore components, likely indicating that they require intact kinetochore structures to localize appropriately. By contrast, peripheral kinetochore proteins were not essential for the correct targeting of either protein. Cells depleted of RanBP2 show abnormalities in both spindle formation and mitotic progression, substantiating the importance of their function during mitosis.

SUMO proteases catalyze the processing of SUMO-1 before the conjugation and deconjugation of SUMO-1 from other proteins. Budding yeast has two SUMO proteases, Ulp1p and Ulp2p/Smt4p. Ulp1p is concentrated near the nuclear periphery and interacts with nuclear pore components, as demonstrated in two-hybrid assays. *ULP1* is an essential gene, and temperature-sensitive Ulp1p mutants arrest at the G2/M transition of the cell cycle. In mammals, there are at least seven SUMO protease family members. We are systematically investigating these enzymes and have previously shown that one mammalian SUMO protease (SENP2) associates with the nuclear pore through an interaction between its N-terminus and Nup153. More recently, our analysis of SENP protease suggests that other proteases also associate with the nuclear pore but use different binding sites. Together with earlier findings regarding the association of conjugating enzymes with the nuclear pore (Ubc9 and RanBP2), our results confirm that the nuclear pore is a critical site of SUMO pathway regulation in the cell.

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Mitotic roles of Ran GTPase

Arnaoutov, Breaux, Kametaka, Quimby, Yun, Dasso

Ran is a small GTPase required for nucleocytoplasmic trafficking, spindle assembly, nuclear assembly, and cell cycle control. The nucleotide exchange factor for Ran, RCC1, is a chromatin-associated protein. The GTPase-activating protein for Ran, RanGAP1, is cytoplasmic during interphase. During mitosis, the bulk of RanGAP1 is broadly distributed, although a significant fraction of RanGAP1 becomes associated with kinetochores (see previous section). Ran-GTP nucleotide hydrolysis also requires a family of accessory proteins. The best-characterized member of this family is mammalian RanBP1, which is distributed to the cytosol during interphase. *In vitro,* RanBP1 increases the rate of RanGAP1-mediated Ran-GTP hydrolysis by about an order of magnitude. RanBP1 also promotes dissociation of Ran-GTP from transport receptors, whose binding would otherwise block RanGAP-mediated GTP hydrolysis. It has been widely hypothesized that the distribution of Ran's regulators modulates local concentrations of Ran-GTP within the cell, spatially directing the many processes in which Ran has been implicated. Ran's primary known effectors are a set of Ran-GTP binding proteins that were originally described as nuclear transport receptors. Ran-GTP binding regulates association between these proteins and their transport cargoes.

Defects in the Ran pathway disrupt both the onset and completion of mitosis, although Ran's function in cell cycle progression had not been clearly distinguished from its roles in nuclear transport and spindle assembly. We were therefore interested in examining more closely Ran's role in mitotic regulation. Mitosis is tightly controlled in eukaryotes by the activity of cyclin B and securin. Both cyclin B and securin are ubiquitinated at the metaphase-anaphase transition by the APC/C E3 ligase, working in association with its activators Cdc20/FZY and Cdh1/FZR. In the presence of misassembled spindles, with kinetochores that are unattached or lack tension from spindle microtubules, the onset of anaphase is delayed through activation of a spindle assembly checkpoint. This checkpoint pathway prevents APC/C^{FZY} activation and thereby stabilizes APC/C^{FZY} substrates. After all the chromosomes have become attached and aligned within the mitotic spindle, the checkpoint is turned off, APC/C^{FZY} becomes active, and anaphase commences. Components of the spindle assembly checkpoint include Mad1, Mad2, Mps1, Bub1, Bub3, BubR1, and CENP-E.

We have examined the role of Ran in regulating mitotic checkpoints using *Xenopus* egg extracts, a well-established model system for checkpoint control. During normal cell cycles in cycling egg extracts, we find that the amount of chromatin-associated RCC1 increases dramatically at the onset of cyclin B destruction. Moreover, moderate levels of exogenous RCC1 protein abrogate mitotic spindle checkpoint arrest and allow cyclin B destruction in extracts containing nuclei plus nocodazole, a microtubule depolymerizing agent. We find that the spindle assembly checkpoint in *Xenopus* is characterized by decreased APC^{FZY} activity and that the addition of RCC1 to extracts with the activated checkpoint restores APC^{FZY} activity to control levels. To determine the mechanism through which RCC1 abrogates checkpoint arrest, we examined the localization of mitotic regulators, including Mad2, CENP-E, Bub1, and Bub3. We found that these proteins are mislocalized away from kinetochores in nocodazole-treated extracts after the addition of high levels of RCC1 protein. Interestingly, displacement of Bub1 and Bub3 from kinetochores could be reversed by the addition of recombinant RanGAP1 protein, suggesting that their behavior responds directly to Ran-GTP levels. Taken together, our results indicate that the Ran pathway is normally regulated in a highly dynamic manner during mitosis. The transitions of the Ran pathway can be mimicked by the addition of exogenous RCC1 protein, triggering the metaphase-anaphase transition prematurely in the presence of unattached kinetochores. Our observations suggest that changes in RCC1's chromosomal dynamics may be a critical link in the chain of events between completion of metaphase spindle assembly and mitotic exit.

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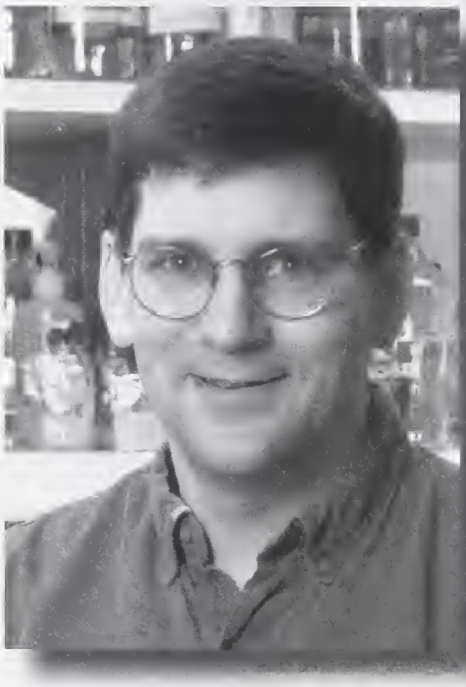
MECHANISM AND REGULATION OF EUKARYOTIC PROTEIN SYNTHESIS



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Chune Cao, *Biological Laboratory Technician*
Joo-Ran Kim, BS, *Special Volunteer*

We are studying the mechanism and regulation of eukaryotic protein synthesis. A major interest is the role of GTPases in translation initiation. We showed that the universally conserved translation initiation factor eIF5B, an ortholog of bacterial translation initiation factor IF2, promotes ribosomal subunit joining in the second GTP-dependent step of the translation initiation pathway. We are using mutational analyses to study the structure/function properties of eIF5B, to determine the role of GTP binding and hydrolysis by the factor, and to study the factor's interaction with the ribosome. We are also analyzing the structure-function properties of the factor eIF2, which binds $tRNA_i^{Met}$ to the ribosome, and are studying how eIF2 activity is down-regulated by phosphorylation. Our studies focus on the determinants of substrate recognition by the eIF2alpha protein kinases and the structure and regulation of these enzymes. The human eIF2alpha kinase PKR is an important component in the interferon-mediated antiviral response. We have reconstituted in yeast cells the antagonism of PKR by pseudosubstrate inhibitors encoded by pox viruses. By identifying mutations in these inhibitory proteins, and others in PKR that increase or decrease inhibition, we are identifying molecular determinants of substrate recognition in both eIF2alpha and PKR.

Molecular, biochemical, and structural analysis of eIF5B/IF2, a universally conserved translation initiation GTPase

Arefin, Cao, Fringer, Kim, Shin, Dever; in collaboration with Burley, Lorsch, Pestova

In bacterial cells, assembly of a functional ribosome at the start codon of an mRNA is facilitated by three initiation factors (IF), whereas at least 12 initiation factors (eIFs) are required in eukaryotic cells. We previously discovered the translation initiation factor eIF5B and characterized it from both yeast (encoded by the *FUN12* gene) and humans. The eIF5B is an ortholog of the bacterial translation initiation factor IF2. In collaboration with Tatyana Pestova, we found that human eIF5B promoted the subunit-joining step of translation initiation. Like bacterial IF2, eIF5B is a GTP-binding protein and possesses ribosome-dependent GTPase activity. In collaboration with Stephen Burley, we determined the X-ray structure of eIF5B from the archaeon *Methanobacterium thermoautotrophicum*. The extended protein is composed of four domains and resembles a chalice. Comparison of the structures of the active (GTP) and inactiv (GDP) forms of eIF5B revealed that modest structural changes in the GTP-binding domain (in the cup of the chalice) are amplified by an articulated lever mechanism resulting in significant movement of domain IV (the base of the chalice). Our current efforts are aimed at elucidating the structure-function properties of eIF5B and molecularly dissecting the role of GTP binding and hydrolysis by the factor.

The discovery of an ortholog of bacterial IF2 in eukaryotes was unexpected. A prominent role of IF2 is to bind the initiator methionyl-tRNA (Met-tRNA) to the ribosome, a role performed by the GTPase eIF2 in eukaryotes. Thus, consensus held that only a single GTP molecule was used during translation initiation in both prokaryotes (IF2) and eukaryotes (eIF2). The discovery of eIF5B led us to predict that two GTP molecules would be consumed during translation initiation in eukaryotes. To test our prediction, we generated a mutant form of human eIF5B that changed nucleotide specificity from GTP to XTP. Ribosome-dependent nucleotide hydrolysis assays confirmed that the mutation eliminated GTPase activity but endowed the factor with XTPase activity. Consistent with the altered nucleotide specificity, the mutant protein catalyzed subunit joining in the presence of XTP, but not GTP. The results demonstrate that two GTP molecules are required in eukaryotic translation initiation (Lee *et al.*, 2002).

To assess the role of GTP binding and hydrolysis by eIF5B, we mutated the Switch 1 (Sw1) motif in the factor's GTP-binding domain. Mutation of a threonine conserved in Sw1 of all GTP-binding proteins eliminated eIF5B GTPase and translational stimulatory activities and severely impaired yeast cell growth. Interestingly, the mutant factor retained the ability to promote subunit joining. Thus, GTP hydrolysis by eIF5B is necessary for protein synthesis but not for subunit joining. Intragenic suppressors were isolated in the eIF5B Sw1 mutant. The suppressor mutations restored yeast cell growth, but not eIF5B GTPase activity. Biochemical studies demonstrated that one of the suppressor mutations, located in the GTP-binding domain of eIF5B, lowered the ribosome-binding affinity of the factor. The uncoupling of translation and eIF5B GTPase activity suggests a regulatory rather than mechanical role for eIF5B GTP hydrolysis in translation initiation. We propose that a GTP-regulated switch governs the ribosome affinity of eIF5B. In the presence of GTP, eIF5B binds to the ribosome, and, following GTP hydrolysis, the factor is released (Shin *et al.*, 2002).

On most mRNAs, ribosomes initiate translation at the AUG codon closest to the 5′ end. However, in yeast lacking eIF5B or expressing eIF5B mutants that lack GTPase activity, we observed an increase in ribosomes' leaky scanning: bypassing the first AUG codon and instead initiating translation at a downstream AUG codon. We propose that the GTPase activity of eIF5B serves as a checkpoint to ensure efficient and accurate subunit joining during translation initiation (Lee *et al.*, 2002; Shin *et al.*, 2002). Thus, when a scanning 40S ribosomal complex reaches an AUG codon, GTP hydrolysis by eIF2 results in release of initiation factors. We propose that eIF5B•GTP binds to the complex, stabilizes Met-tRNA binding, and promotes joining of the large ribosomal subunit. Upon 60S joining, eIF5B hydrolyzes GTP, and the factor is released, enabling the 80S ribosome to begin translation elongation.

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Pox virus pseudosubstrate inhibitors of eIF2alpha kinases: molecular implications for kinase–substrate interactions

Liu, Cao, Dever; in collaboration with Kennedy, Ramelot

Phosphorylation of the translation factor eIF2alpha on serine at residue 51 (Ser-51) by the kinase

PKR is an important component in the antiviral defense mechanism in mammalian cells. Several pox viruses express small proteins resembling the N-terminal third of eIF2alpha, and earlier work demonstrated that the vaccinia virus K3L protein is a pseudosubstrate inhibitor of eIF2alpha kinases. Previously, we showed that expression of either the vaccinia virus K3L or the swinepox virus C8L protein reduces eIF2 alpha phosphorylation and blocks the toxic effects associated with expression of PKR in yeast. This inhibition of PKR by the K3L and C8L proteins was dependent on a sequence motif KGYID conserved among all K3L homologs and eIF2alpha and located about 30 residues C-terminal of the Ser-51 phosphorylation site in eIF2 alpha. We collaborated with Theresa Ramelot and Michael Kennedy to characterize the K3L homolog from myxoma virus, the M156R protein. The NMR structure of the M156R protein revealed that the critical KGYID sequence motif and the region corresponding to Ser-51 in eIF2alpha are located on the same face of the M156R protein, likely forming the PKR recognition surface. In contrast to the K3L protein, we demonstrated that the M156R protein is an efficient *in vitro* substrate for phosphorylation by PKR. Mutational analyses indicated that the primary site of phosphorylation is a tyrosine residue located in the position corresponding to Ser-51 in eIF2alpha. Furthermore, the M156R protein competed with eIF2alpha for phosphorylation by PKR. Thus, rather than functioning as a pseudosubstrate inhibitor like the K3L protein, the M156R protein may interfere with eIF2alpha phosphorylation through simple substrate competition (Ramelot *et al*., 2002).

To gain further insights into pseudosubstrate inhibition of PKR, we are employing two approaches. First, we are characterizing K3L mutants that are more potent inhibitors of PKR. Expression of wild-type K3L partially suppresses the toxic effects of PKR in yeast. We identified several hyperactive K3L mutants that more effectively suppress PKR toxicity in yeast. In addition, we identified PKR mutants that are resistant to inhibition by K3L. These PKR mutants are toxic in yeast strains coexpressing the K3L protein and likewise retain the ability to phosphorylate eIF2alpha, even in the presence of the viral pseudosubstrate. To date, we have identified 12 single amino acid changes in PKR that render the kinase resistant to inhibition by the K3L protein. Interestingly, the mutations cluster in the C-terminal lobe of the kinase domain; based on structural modeling, we conclude that the mutations likely alter points of contact between PKR and its pseudosubstrate (or substrate). Ongoing experiments seek to determine how the mutations in PKR render the kinase less sensitive to pseudosubstrate inhibition.

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Substrate recognition by the eIF2alpha protein kinases

Dey, Cao, Dever

In addition to PKR, three other kinases are known to phosphorylate eIF2alpha on Ser-51. The additional kinases are activated under various cellular stress conditions and down-regulate protein synthesis during stress. The kinase GCN2 is universally conserved in eukaryotes and phosphorylates eIF2alpha under amino acid–limiting conditions. To gain insights into the mechanism of substrate recognition by the eIF2alpha kinases GCN2 and PKR, we generated N- and C-terminal truncation mutants of yeast eIF2alpha to define the minimal substrate that is efficiently phosphorylated. Residues 1 to 180 of eIF2alpha were necessary and sufficient for efficient phosphorylation of Ser-51 both *in vivo* and *in vitro*. Deletion of as few as 10 N- or C-terminal residues from the eIF2alpha 1 to 180 protein severely impaired phosphorylation of Ser-51. The results from these studies together with the results from our studies on the K3L protein indicate that GCN2 and PKR recognize a large domain of eIF2alpha. In addition, our results demonstrate that kinase-substrate recognition for eIF2alpha phosphorylation is not limited to a short consensus sequence element flanking the phosphorylation site, as demonstrated for other kinases.

Phosphorylation of eIF2alpha impairs protein synthesis by converting eIF2 from a substrate to an inhibitor of its guanine-nucleotide exchange factor eIF2B. To define specifically the eIF2alpha determinants required for Ser-51 phosphorylation and translational regulation, we randomly mutated each of the six residues that flank Ser-51 plus the residues in the conserved KGYID motif located at residues 79 to 83. Few substitutions in the residues flanking Ser-51 impaired phosphorylation; however, many mutations at these residues altered translational regulation. Thus, the eIF2alpha residues flanking Ser-51 are critical for inhibition of eIF2B by phosphorylated eIF2. In contrast, several mutations in the KGYID motif significantly impaired phosphorylation of Ser-51. Of particular note, substitution of Ala for Asp-83, 32 residues from the phosphorylation site at Ser-51, completely blocked eIF2alpha phosphorylation by GCN2 and PKR both *in vivo* and *in vitro*. Interestingly, several mutations in the KGYID motif impaired translational regulation without affecting Ser-51 phosphorylation. Thus, the eIF2alpha residues flanking Ser-51 and in the KGYID motif contribute to both kinase recognition and eIF2B inhibition, indicating that the eIF2alpha kinases and eIF2B recognize overlapping surfaces on eIF2alpha. The novel requirement for specific residues remote from the site of phosphorylation supports the model that the eIF2alpha kinases recognize a large domain of eIF2alpha. The critical importance of remote sequences for eIF2alpha phosphorylation, combined with the ease of conducting *in vivo* analyses of kinase and substrate mutants in yeast, establishes the yeast mutants as ideal for studying protein kinase-substrate recognition.

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TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL MECHANISMS IN NUTRIENT REGULATION OF GENE EXPRESSION

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We are studying regulatory mechanisms in the yeast *Saccharomyces cerevisiae* that stimulate transcription of amino acid, vitamin, and purine biosynthetic genes in response to nutrient limitation. Translation of transcriptional activator GCN4 is stimulated in starved cells by a mechanism involving short open reading frames (uORFs) in the mRNA leader and phosphorylation of initiation factor eIF2. Bound to GTP, eIF2 delivers initiator $tRNA_i^{Met}$ to the 40S ribosome. Phosphorylation of eIF2 by the kinase GCN2 inhibits formation of the eIF2-GTP-$tRNA_i^{Met}$ ternary complex (TC), reducing general protein synthesis but stimulating translation of GCN4. We are analyzing the physical and functional interactions of eIF2 with other initiation factors (eIFs-1, -1A, -3, and -5) and the 40S ribosome that promote TC recruitment, ribosomal scanning, and recognition of AUG codons during general and GCN4-specific translation. We are also studying the regulation of GCN2 kinase activity by uncharged tRNA (the starvation signal), a protein that associates with GCN2 on translating ribosomes (GCN1), and the TOR signaling pathway. Finally, we are analyzing the coactivators required for transcriptional activation by GCN4 to define the molecular program for the recruitment of chromatin-remodeling enzymes and adaptor proteins that deliver TATA-binding protein and RNA polymerase to target genes *in vivo*.

Analysis of eIF2-eIF3 contacts and ribosome-binding domains in the multifactor complex that are required for general and GCN4-specific translation

Valasek, Nielsen, Szamecz, Jivotovskaya, Hinnebusch

According to current models, the TC binds to the 40S ribosome with the help of eIFs 1, 1A, and 3. Interaction of the 43S preinitiation complex thus formed with mRNA is stimulated by eIF4F and poly(A)-binding protein, with the resulting 48S complex scanning the mRNA until the Met-$tRNA_i^{Met}$ base-pairs with the AUG start codon. On AUG recognition, the eIF5 stimulates GTP hydrolysis by eIF2, the eIFs are ejected, and the 60S subunit joins with the 40S-Met-$tRNA_i^{Met}$-mRNA complex in a reaction stimulated by eIF5B. We are probing the relative importance of eIFs-1, -1A, and -3 in the recruitment of TC and mRNA to the 40S ribosome, scanning, and AUG selection by generating mutations in these factors and examining the consequences on the rate of translation initiation, 43S/48S complex assembly, and *GCN4* translational control in living cells.

We previously showed that eIF3 contains five subunits and exists in a multifactor complex (MFC) with eIFs-1, -5, and the eIF2-GTP-Met-tRNA$_i^{Met}$ ternary complex, produced a detailed subunit-interaction map for the MFC components, and showed that MFC integrity depends on simultaneous interaction of the eIF5 C-terminal domain (CTD) with eIF1, eIF3c/NIP1, and eIF2-beta. Furthermore, we showed that disrupting these interactions by the *tif5-7A* mutation in the eIF5-CTD impairs translation initiation and cell growth. To obtain further support for our model, we incorporated an affinity tag into TIF32, PRT1, and NIP1, the three largest eIF3 subunits, and deleted predicted binding domains in each tagged protein. We confirmed all binding interactions predicted by our model and uncovered a new interaction between the TIF32 CTD and eIF2-beta that is required for eIF2-eIF3 association. Overexpressing a CTD-less form of TIF32 exacerbates the translation initiation defect of the *tif5-7A* mutation and reduces eIF2 binding to 40S subunits, which implies that the two eIF2-eIF3 contacts in the MFC additively enhance the efficiency of TC recruitment and the rate of translation initiation (Valasek *et al.*, 2002).

Reducing the rate of TC binding to 40S ribosomes is expected to derepress *GCN4* translation (Gcd$^-$ phenotype), which we observed for a mutant lacking the CTD of eIF1A (Olsen *et al.*, 2003). By contrast, we did not observe a Gcd$^-$ phenotype in *tif5-7A* cells overexpressing CTD-less TIF32, despite the reduction in levels of 40S-associated TC, indicating that eIF2-eIF3 contacts in the MFC also contribute to functions downstream of TC recruitment, such as scanning and AUG recognition, and that defects in these processes suppress the effects of reduced impaired TC recruitment on *GCN4* translation. We recently obtained additional support for our conclusion by showing that mutants with conditionally lethal mutations in eIF3 subunits accumulate 48S preinitiation complexes containing TC, mRNA, and all relevant eIFs under nonpermissive conditions that impair translation initiation. Thus, it appears that eIF3 is critical for one or more post-assembly functions of the 48S complex. We are testing this hypothesis further by examining the effects of eIF3 mutations on *GCN4* translational control and the stringency of AUG selection during the scanning process.

Having previously characterized MFC subcomplexes produced from affinity-tagged eIF3 subunits lacking discrete binding domains for other MFC components, we investigated whether these subcomplexes can bind to 40S ribosomes *in vivo*. The N- and C-terminal domains of NIP1/eIF3c, the N- and C-terminal domains of TIF32/eIF3a, and eIF5 all have critical functions in 40S binding, with eIF5 and the TIF32-CTD performing redundant functions. In accordance with these results, purified eIF3 and a trimeric complex consisting of NIP1, C-terminally truncated TIF32, and eIF5 can bind to purified 40S ribosomes *in vitro*. Given that removing the TIF32-NTD and NIP-CTD does not disrupt the MFC, these domains likely make direct contacts with the 40S ribosome. Furthermore, the TIF32-CTD interacts specifically *in vitro* with helices 16 to 18 of domain I in 18S rRNA, and the TIF32-NTD and NIP1 can interact with recombinant 40S subunit protein RPS0A. These results, together with the known positions of helices 16 to 18 and RPS0A in the three-dimensional structure of the 40S subunit, suggest that eIF3 binds to the solvent side of the 40S subunit in a way that provides access for the two eIF3 segments (NIP1-NTD and TIF32-CTD) with connections to eIFs 1, 5, and the eIF2/GTP/Met-tRNA$_i^{Met}$ ternary complex (Valasek *et al.*, 2003) (see Figure 13.1).

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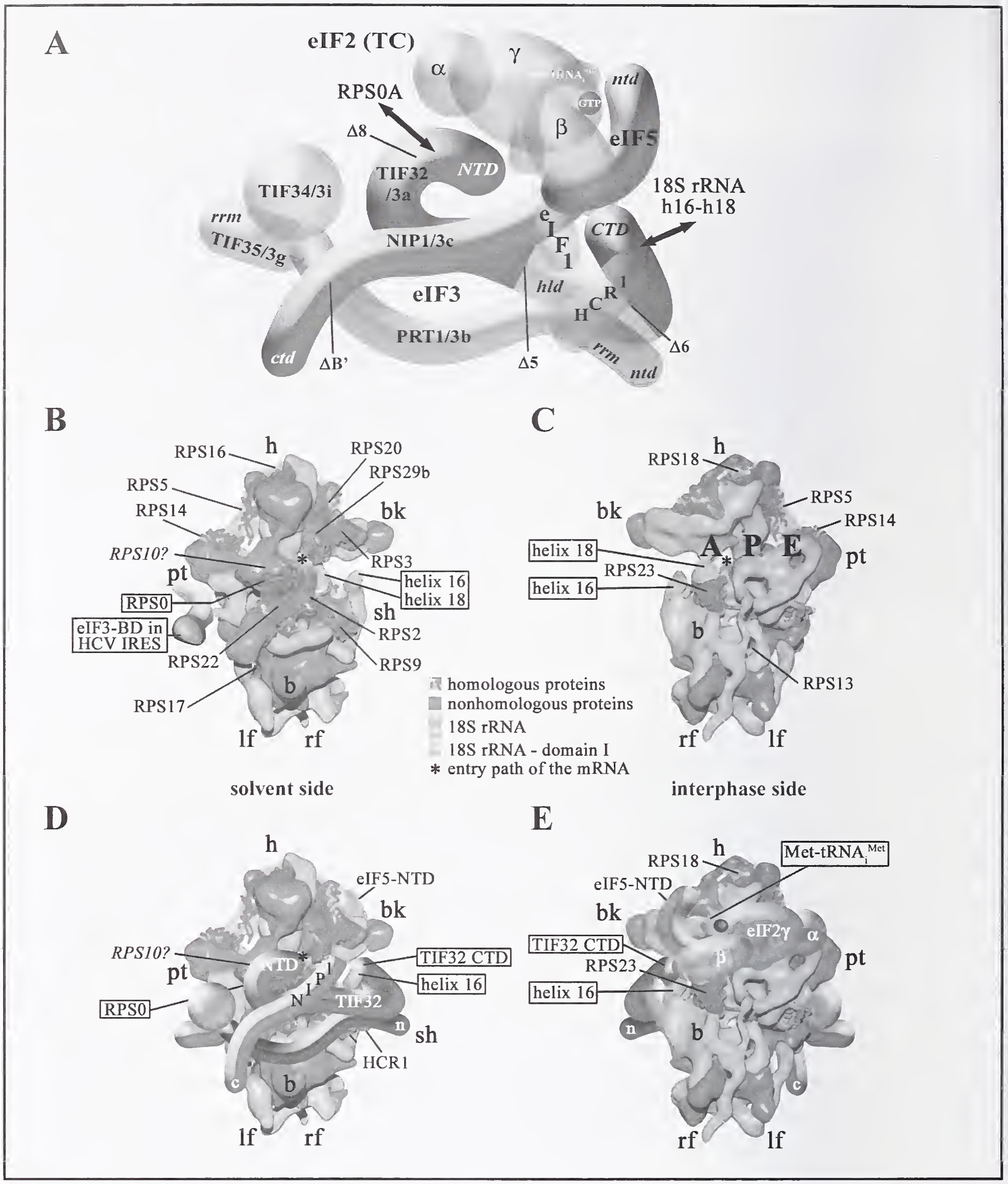

FIGURE 13.1 ***Model*** **predicting the interaction of eIF3 with the** ***S. cerevisiae*** **40S ribosomal subunit.**

(A) A 3-D model of the MFC based on a comprehensive analysis of subunit interactions (Valasek *et al.* 2002). The labeled protein subunits are shown roughly in proportion to their molecular weights. The degree of overlap between two different subunits depicts the extent of their interacting surfaces. The boundaries of relevant deletions truncations are indicated at the appropriate positions in TIF32 and NIP1. ntd, N-terminal domain; ctd, C-terminal domain; hld, HCR1-like domain; rrm, RNA recognition motif. The segments with solid shading represent the domains with a critical role in association of the MFC with the 40S ribosome. Interactions of the TIF32-NTD with RPS0A and of TIF32-CTD with helices 16-18 of 18S rRNA are indicated by arrows. (B-C) Cryo-EM reconstruction of the *S. cerevisiae* 40S subunit docked with modified atomic models of 18S rRNA and 40S ribosomal proteins (adapted from Spahn *et al. Cell* 2001;107:373-386). The 40S subunit is shown from the solvent (B) or interface (C) sides. The ribbon models of the proteins with homology to *E. coli* small ribosomal proteins are shown in a transparent envelope and labeled and boxed (RPS0). The solid protein segments are predicted to be composed of proteins without prokaryotic homologs. Domain I of 18S rRNA is shown with the positions of helices 16 and 18 indicated. (B) Position of the eIF3-binding domain in the HCV IRES bound to the small subunit is indicated

Valasek L, Nielsen KH, Hinnebusch AG. Direct eIF2-eIF3 contact in the multifactor complex is important for translation initiation in vivo. *EMBO J* 2002;21:5886-5898.

The yeast TOR proteins impede activation of GCN2 in nutrient-replete cells by promoting phosphorylation of Ser577

Cherkasova, Garcia-Barrio, Dong, Zhang, Hinnebusch; in collaboration with Qin*

Through mass-spectrometric analysis of purified, catalytically inactive GCN2, we identified Ser577 as a site of phosphorylation by another kinase *in vivo*. It appears that Ser577 phosphorylation inhibits GCN2 because the substitution with alanine (*S577A* mutation) results in constitutive eIF2alpha phosphorylation and derepressed *GCN4* translation. The *S577A* mutation increases the affinity of GCN2 for uncharged tRNA *in vitro*, suggesting that Ser577 phosphorylation impedes GCN2 activation in rich medium by preventing the binding of uncharged tRNAs present at basal concentrations in nonstarved cells (Garcia-Barrio *et al*., 2002).

Because Ser577 remains phosphorylated in amino acid–starved cells, we asked whether it is dephosphorylated in other conditions under which GCN2 is activated. We found that activation of GCN2 by the antibiotic rapamycin is associated with dephosphorylation of Ser577. The *S577A* mutation dampened the effect of rapamycin on eIF2alpha phosphorylation and *GCN4* translation, consistent with the idea that activation of GCN2 by rapamycin requires dephosphorylation of Ser577. The stimulatory effect of rapamycin requires tRNA binding by GCN2, suggesting that rapamycin increases the affinity of GCN2 for uncharged tRNA through Ser577 dephosphorylation, allowing kinase activation by the basal levels of uncharged tRNA present in nutrient-replete cells (Cherkasova and Hinnebusch, 2003). Our genetic analysis indicates that rapamycin stimulates eIF2alpha phosphorylation by GCN2 through the inhibition of TOR1 and TOR2, two PI3-kinase–related proteins that stimulate protein synthesis and repress pathways for utilization of poor nitrogen sources in nutrient-rich medium. We demonstrated that, in yeast, TOR proteins inhibit GCN2 function rather than stimulating the eIF2alpha phosphatase. In addition, we showed that rapamycin-induced dephosphorylation of GCN2-Ser577 involves TAP42, a regulator of type 2A–related protein phosphatases (PPases) implicated previously as a regulator of certain targets of the TOR pathway. Thus, it seems likely that TOR promotes GCN2-Ser577 phosphorylation in nutrient-replete cells by inhibiting the dephosphorylation of this residue by a type 2A–related PPase (Cherkasova and Hinnebusch, 2003) (see Figure 13.2).

Our results add a new dimension to the regulation of protein synthesis by TOR proteins and demonstrate cross-talk between two major pathways for nutrient control of gene expression. The TOR pathway stimulates translation in yeast by promoting ribosome biogenesis and the abundance of mRNA cap–binding factor eIF4F. We show that TOR also stimulates eIF2 function by preventing activation of GCN2. In addition to enhancing protein synthesis, inhibition of GCN2 by TOR maintains repression of *GCN4* and the amino acid–biosynthetic genes under its control in nutrient-replete cells. Interestingly, the derepression of *GCN4* that occurs when TOR is inhibited by rapamycin augments the transcriptional induction of genes required for utilization of poor nitrogen sources by the activator protein GLN3.

Figure 13.1 Legend (continued)

(adapted from Spahn *et al. Science* 2001;291:1959-1962). (D-E) Hypothetical location of the eIF3 complex on the 3-D model of the 40S subunit, explaining the requirements for the N- and C-terminal domains in both NIP1 and TIF32 for 40S binding, interaction between the TIF32-NTD and RPS0A, binding of the TIF32-CTD to helices 16 to 18 of 18S rRNA, and binding of NIP1 to RPS0A and 18S rRNA. The other MFC components, eIFs 1, 2, and 5, which are tethered to eIF3 via the NIP1-NTD and TIF32-CTD, are predicted to interact with the interface side of the 40S subunit, as shown for a small portion of eIF5 visible from the solvent side in (D) and for eIF5 and the Met-tRNA$_i^{Met}$ eIF2–GTP TC in (E). The dimensions of the MFC model relative to the dimensions of the 40S subunit were determined from the molecular weights of both macromolecules, from previously published EM reconstitutions of the eIF3–40S complex and from the relative dimensions of the 3-D structures of the 30S ribosomal subunit of *Thermus thermophilus* and eIF2alpha obtained from the Protein Data Bank.

Figure 13.2 Legend

Model depicting the TAP42/Ser577-dependent pathway and a partially redundant uncharacterized pathway utilized by TOR to down-regulate GCN2 activity when nitrogen is plentiful. Phosphorylation of Ser577 inhibits the GCN2 protein kinase (PK) domain. Treatment with rapamycin inhibits TOR, leading to dephosphorylation of Ser577 by the TAP42-regulated phosphatases SIT4/PP2A. The ensuing activation of GCN2 and eIF2alpha phosphorylation down-regulates general translation initiation and induces GCN4 translation. The GCN4 thus produced activates amino acid biosynthetic genes and augments the GLN3-dependent activation of genes required for catabolism of poor nitrogen sources. Because rapamycin can stimulate eIF2alpha phosphorylation in tap42-11 cells at 25°C even though Ser577 remains phosphorylated, we propose that a redundant pathway exists for TOR-dependent inactivation of GCN2 that is also inhibited by rapamycin. Starvation for amino acids can lead to activation of GCN2 through binding of uncharged tRNA to the HisRS domain even under conditions of TOR-dependent Ser577 phosphorylation.

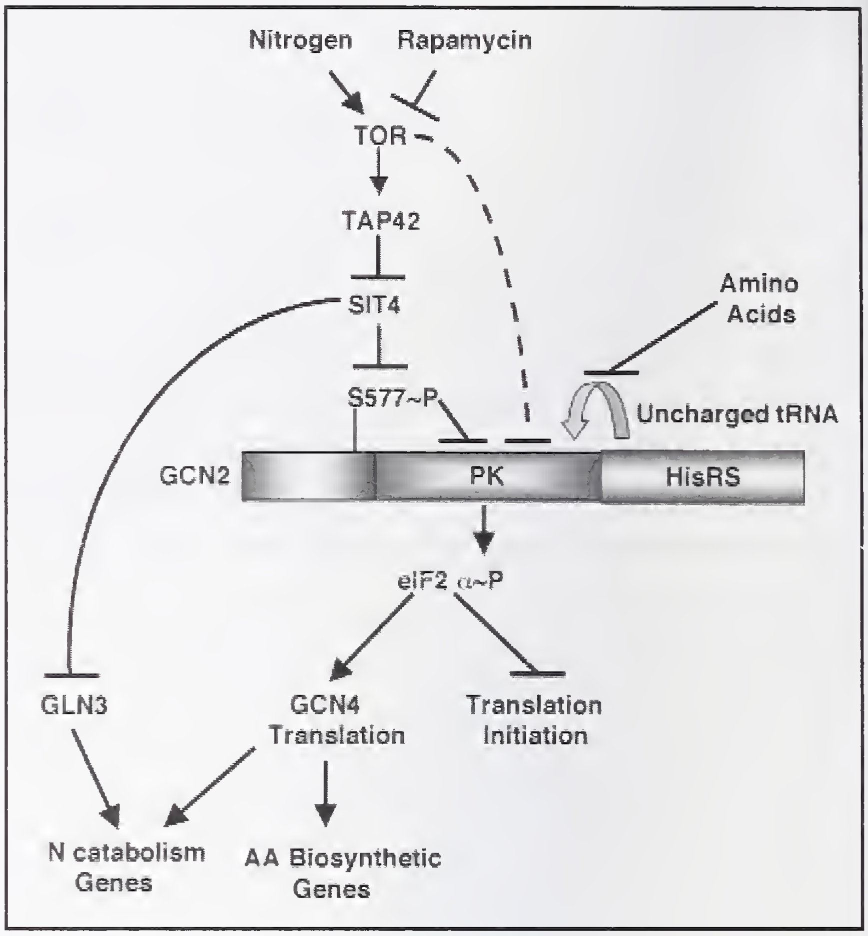

FIGURE 13.2

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A multiplicity of coactivators required for transcriptional activation by GCN4; requirements for SWI/SNF recruitment by GCN4 *in vivo*

Swanson, Qiu, Yoon, Sumibcay, Kim, Zhang, Hu, Hinnebusch

Transcriptional activation in eukaryotes typically involves sequence-specific DNA binding proteins that bind upstream of promoters and recruit multisubunit coactivator complexes with the capacity to stimulate assembly of a preinitiation complex (PIC) at the promoter. Some coactivators, including SWI/SNF and RSC, are ATP-dependent enzymes capable of remodeling the nucleosome structure of the promoter while others, such as the SAGA complex, contain histone acetyltransferase (HAT) activities that facilitate chromatin remodeling or mark promoter nucleosomes as binding sites for other coactivators. A third class of coactivators, including the Srb and Paf1 mediators, TFIID, and CCR4-NOT, are physically associated with TATA-binding protein (TBP), other general transcription factors (GTFs), or RNA polymerase II (Pol II) and are thought to function as adaptors between the activator and transcriptional machinery that promote PIC assembly. Our previous studies showed that SAGA, SWI/SNF, and Srb mediator complexes can interact specifically with GCN4 *in vitro*, dependent on bulky hydrophobic residues in the GCN4 activation domain that are required for transcriptional activation *in vivo*. In addition, we and others showed that mutations in various subunits of these three coactivators reduce activation by GCN4 *in vivo* (Gcn$^-$ phenotype).

More recently, we conducted a comprehensive analysis of coactivator requirements by testing all viable mutants from the *Saccharomyces* Deletion Project for defects in activation by GCN4 *in vivo*.

Our data confirm that GCN4 requires SAGA, SWI/SNF, and SRB/MED and identify the critical nonessential subunits in these complexes required for activation *in vivo*. Among the numerous histone acetyltransferases examined, GCN5 was the only one required for activation by GCN4. We also uncovered a strong dependence on the CCR4-NOT complex and significant requirements for RSC and the Paf1 complex in activation by GCN4 but found no role for the ISW1 and ISW2 chromatin remodeling complexes. We showed that the GCN4 activation domain interacts specifically with CCR4-NOT and RSC in addition to SAGA, SWI/SNF, and SRB/MED, but not with the Paf1 complex, whereas GCN4 recruits all six of these coactivators, plus the TBP adaptor protein MBF1, to the same target gene (*ARG1*) in living cells. Thus, GCN4 recruits an array of coactivators that promote its transcriptional activation function *in vivo* (Swanson *et al*., 2003).

We wished to determine the roles of different subunits of SWI/SNF and the coactivators SAGA and Srb mediator in recruitment of SWI/SNF by GCN4 *in vivo*. To this end, we performed ChIP analysis on deletion mutants lacking different subunits of these coactivators and bearing epitope tags on six different SWI/SNF subunits. The results provide strong evidence that Gcn4p recruits the entire SWI/SNF complex to its target genes *ARG1* and *SNZ1* but that SWI/SNF is dispensable for GCN4 binding to these promoters. Previous work showed that the SWI/SNF subunits SNF2, SNF5, and SWI1 interact directly with GCN4 *in vitro*. However, we found that SNF2 is not required for recruitment of SWI/SNF by GCN4 and that SNF2 cannot be recruited independently of other SWI/SNF subunits *in vivo*. SNF5 also was not recruited as an isolated subunit but was required along with SNF6 and SWI3 for optimal recruitment of other SWI/SNF subunits. The results suggest that SNF2, SNF5, and SWI1 are recruited only as subunits of intact SWI/SNF, consistent with the idea that GCN4 makes multiple contacts with SWI/SNF *in vivo*. Optimal recruitment of SWI/SNF by GCN4 also requires specific subunits of the Srb mediator (GAL11, MED2, ROX3) and SAGA (ADA1 and ADA5) but is independent of the histone acetyltransferase in SAGA, GCN5. Based on these findings, we suggest that SWI/SNF recruitment is enhanced by cooperative interactions with subunits of Srb mediator and SAGA that are recruited by GCN4 to the same promoter but is insensitive to histone H3 acetylation by GCN5 (Yoon *et al*., in press). Experiments are now under way to probe the requirements for the recruitment of SAGA, Srb mediator, TBP, and Pol II by GCN4 and to define the kinetic order of recruitment of these factors and coactivators at various target genes *in vivo*.

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Naoe Kotomura, PhD, *Guest Researcher*

Our research focuses on understanding the mechanisms by which entire regions of the genome are rendered inaccessible to transcription and recombination. We undertake genetic analysis coupled with biochemical fractionation and reconstitution experiments to explore the issues of genome accessibility. Silencing of genomic domains requires a complex series of interactions between inactivation centers called silencers and numerous repressor proteins. The silencers recruit repressor protein complexes composed of the Sir proteins that interact with histones in nucleosomes to form a chromatin domain that is both inaccessible and inert to various cellular processes. We are currently focusing on the Sir proteins and their interactions with the histones to understand in molecular detail the mechanism by which silencing is effected.

Histone variants and cell cycle progression

Dhillon

There are numerous histone variants that vary in amount and localization and whose expression is regulated, suggesting a role in gene regulation. We isolated Htz1p, a histone variant, as a suppressor of a Sir1p mutant, and recent results suggest that the protein is localized to regions flanking the silenced loci. We are currently interested in understanding the many roles of the protein in the cell. Cells lacking this protein are hypersensitive to various drugs such as hydroxyurea and benomyl. We have generated point mutations in the protein that are sensitive to these drugs and find that they all map to a single domain. Suppressor analyses of the point mutants are under way and should help identify proteins interacting with this domain. We also observe that cells lacking the protein have defects in cell cycle progression, and genetic data suggest that lack of the protein elicits specific checkpoints. We are currently attempting to ascertain the nature of the cell cycle defect to gain a better understanding of the function of the protein in the cell.

Dhillon N, Kamakaka RT. A histone variant Htz1p and a Sir1-like protein Esc2p mediate silencing at *HMR*. *Mol Cell* 2000;6:769-780.

Identification and characterization of Sir protein complexes involved in silencing

Gangadharan, Kotomura

We are interested in determining how Sir proteins function to form silenced domains. Genetic studies have revealed that distinct combinations of the Sir protein complexes repress multiple loci. Silencing at all these loci requires Sir2p, which possesses histone deacetylase activity. Furthermore, Sir2p is the only Sir protein to have additional homologs in yeast (Hst1-4p) and the only Sir protein conserved throughout evolution. We are purifying Sir2p-containing protein complexes from yeast cells to identify and characterize individual components within these complexes and their associated enzymatic activities. We have purified two Sir2p-containing complexes, the TEL and RENT complexes, and have identified a second deacetylase in the RENT complex. By using reverse genetics and experiments to analyze the nature of the interactions between the proteins, we are determining the role of this deacetylase in silencing.

Using the purified complexes and histones in nucleosomes (Ghidelli *et al.*, 2001), we have also initiated studies on the reconstitution of silenced chromatin. We are performing *in vitro* binding studies with purified Sir complexes as well as with recombinant Sir proteins with positioned nucleosomes in arrays, followed by DNaseI foot-printing analysis and protein-protein cross linking and sedimentation analysis. Over the long term, we plan to undertake studies on the regulation of these enzymes within the cell and *in vitro* studies aimed at the eventual development of specific inhibitors of these enzymes that may have therapeutic value. Given that mechanisms are rarely established by genetic means and usually require biochemical tests, our studies will provide an important index of our current understanding of transcriptional silencing depending on whether or not it is possible to mimic the silenced state *in vitro* exactly.

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Chromatin domains in silencing

Oki, Valenzuela; in collaboration with Ito

We are also interested in understanding the mechanism by which silenced chromatin domains are restricted to specific regions along the DNA fiber. Eukaryotic chromosomes are organized into discrete domains delimited by domain boundaries. We have demonstrated that a specific t-RNA gene mediates barrier functions at the *HMR* locus. The proteins that are required to prevent the spread of heterochromatin into neighboring euchromatin have also been identified (Donze and Kamakaka, 2000). Our results suggest that barrier activity may arise from an underlying competition between chromatin remodeling and silencing activities at the interface of euchromatin and heterochromatin. In our ongoing studies on yeast barriers, we have used genetic screens to isolate other DNA elements from yeast and other eukaryotes that act as barrier elements as well as to identify additional proteins from yeast and other eukaryotes that possess barrier activity. These studies will help to delineate general principles of barrier activity in all eukaryotes.

Given that these repressors occupy entire chromatin domains and modify histones within the domains, we are also interested in determining the chromatin structure and modification state of the histones within the repressed domain as well as the adjoining active chromatin to catalog all the differences between active and inactive domains as well as the interplay between these adjoining domains.

Donze D, Kamakaka RT. RNA polymerase III and RNA Polymerase II promoter complexes are heterochromatin barriers in *Saccharomyces cerevisiae. EMBO J* 2001;20:520-531.

Analysis of Sir2p in other eukaryotes

Haldar

A complete understanding of transcriptional repression requires the analysis of several unrelated loci in different and distinct systems so that salient principles of repression can be distinguished from organism- and locus-specific variation. Silencing of chromatin domains in *S. pombe* shows many similarities with heterochromatin formation and position effect variegation in other eukaryotes such as *S. cerevisiae* and *Drosophila*. Functional homologs of the various *S. cerevisiae* genes that affect repression are undergoing identification in *S. pombe* to allow the purification and characterization of protein complexes containing these proteins. We will couple the work on identification with mechanistic studies on nucleosomal binding in this organism.

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MOLECULAR MECHANISM OF NEURONAL CONNECTIVITY

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Using the *Drosophila* visual system as a model, we focus on understanding the molecular mechanisms that direct neuronal connections during development. We are currently studying first-order connectivity, specifically the connections made by the R7 class of photoreceptor neurons to their brain target neurons. We use genetic analysis coupled with confocal microscopy imaging and behavioral assays to explore the mechanisms by which cadherin-mediated adhesion regulates the formation of specific connections. We have previously established that neuronal cadherin is required for R7 target selection. Using a genetic method that allows single-cell mosaic analysis at different developmental stages, we demonstrated that N-cadherin functions in both the initial target recognition process as well as in maintenance of the afferent-target interaction. In addition, we identified a second neuronal cadherin (N-cadherin-2) that most likely resulted from *N-cadherin* gene duplication. Both the *N-cadherin* and *N-cadherin-2* loci encode multiple isoforms (or variants) via pre-mRNA alternative splicing. The N-cadherin isoforms share the same molecular architecture but have divergent sequences in their extracellular and transmembrane domains. In contrast, the N-cadherin-2 variants have different lengths in the transmembrane and cytoplasmic region. We are examining how the molecular diversity of N-cadherins contributes to the logic of brain wiring.

Modular diversification of N-cadherin generated by alternative splicing

Yonekura, Chung, Lee; in collaboration with Chiba, Hsu

Calcium-dependent cell adhesion receptors, or cadherins, have been proposed to mediate synaptic target recognition and synaptogenesis in vertebrate brains. In particular, cadherins are thought to provide the interaction between pre- and postsynaptic partners. Although it is generally accepted that the adhesion activity of cadherins is essential for these functions, the mechanism of action has been in dispute. Cadherins are capable of mediating homotypic and, in some cases, heterotypic interaction. It remains unclear how the synaptic specificity can be attributed to the binding specificity of different cadherins and how cadherin-mediated interactions are regulated. Furthermore, it was recently found that the vertebrate protocadherin CNR (cadherin-related neuronal receptor) and insect N-cadherins can expand their receptor repertoire by alternative splicing. The alternatively spliced cadherin isoforms could largely increase the complexity and specificity of the adhesive interaction in the developing nervous system.

We have previously identified N-cadherin for its requirement in R7 target selection. Genomic sequence inspection and RNA transcript analysis reveal that the *N-cadherin* locus contains three "exon modules," each containing two alternatively used exons (exon 7/exon 7p, exon 13/exon 13p, and exon 18/exon 18p). Using RT-PCR analysis, we identified eight N-cadherin isoforms that are generated by the combinatorial use of these exon pairs. The predicted eight N-cadherin isoforms share the same molecular architecture but have divergent amino acid sequences in the extracellular and transmembrane regions. Semiquantitative RT-PCR analysis indicates that the exons are utilized differentially in a tissue-specific manner. Exon 7 and exon 13 are predominantly used (over 85 percent) in both eye tissue and whole animals. However, exon 18P is the predominant form in the developing eye tissue while, in whole animals, both exons 18 and 18P are found with approximately the same frequency.

All N-cadherin isoforms have the same modular structure, including 16 cadherin repeats in the extracellular domain, a transmembrane region, and a cytoplasmic tail. Exons 7 and 7p encode the carboxyl-terminal half of the eighth cadherin repeat, the linker region, and the amino-terminal half of the ninth cadherin repeat while exons 13 and 13p encode the 11th and 12th cadherin repeats in similar fashion. Molecular modeling using the known E-cadherin structure as a template reveals that amino acid sequences divergent among the isoforms mapped to two distinct regions. The first region mediates calcium-binding and the second has been implicated in homophilic interaction, suggesting that the different N-cadherin-1 isoform might mediate differential homo- or heterophilic interaction. We expressed these isoforms in *Drosophila* S2 cells and tested their ability to induce cell aggregation. Our data indicate that isoforms encoded by exon 7 but not by exon 7p are able to mediate both homo- and heterophilic interaction. These interactions are calcium-dependent, as removing calcium from the medium abolishes cell aggregation. Furthermore, the interaction is type-specific in that the N-cadherin-1 isoforms can interact only with one another but not with E-cadherin, another classical *Drosophila* cadherin.

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N-cadherin-2 locus encodes multiple isoforms of various lengths

Yonekura, Lee; in collaboration with Clandinin, Prakash

Inspection of the genomic sequence close to the *N-cadherin-1* locus has led to the discovery of *N-cadherin-2*, highly homologous to *N-cadherin*. The *N-cadherin-2* gene is smaller than the *N-cadherin-1* gene and is likely a result of a gene duplication event. As genomic sequence analyses failed to resolve the gene structure unambiguously, we performed detailed RNA transcript and RT-PCR analysis on the *N-cadherin-2* gene. We uncovered four types of alternative transcripts at the 5′ end of the mRNA and three types at the 3′ end. Thus, by combinatorial use of these variations, the *N-cadherin-2* locus can potentially generate 12 different transcripts that are translated into six different protein variants. Unlike *N-cadherin-1*, the alternative splicing of *N-cadherin-2* results in protein variants with three different molecular architectures. The variants include one receptor type that is similar to but shorter than the N-cadherin-1, another with a truncated cytoplasmic region, and the third, a secreted form (see Figure 13.3). All the N-cadherin-2 variants contain six cadherin repeats in the extracellular domain. Using cell-based arrays, we are investigating the biological function of the variants. In addition, we will examine the *N-cadherin-2* phenotype in R7 target selection.

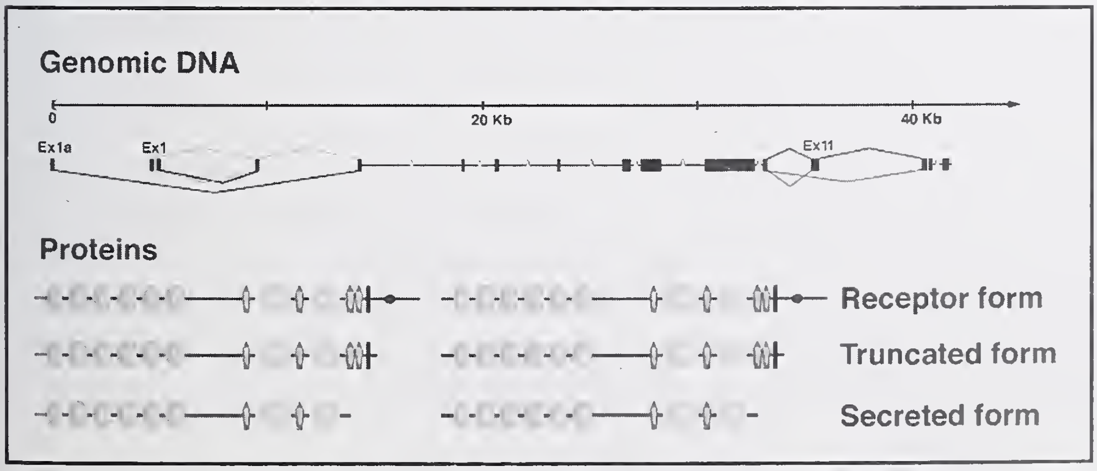

FIGURE 13.3

N-cadherin-2 locus encodes six isoforms of various lengths.

An N-cadherin gene family in *Anopheles gambiae* uncovered by comparative genomic analysis

Lee

To gain insight into the biological significance of N-cadherin molecular diversity, we performed comparative genomic analysis in a mosquito and another member of the *Drosophila* family. *Drosophila melangaster* diverged from the malaria mosquito (*Anopheles gambiae*) and *Drosophila pseudoobscura* about 250 and 25 million years ago, respectively. We identified a parallel modular organization in the *N-cadherin* ortholog of both mosquito and *pseudoobscura*. In addition, the amino acid sequences specific to the alternative exons are mostly preserved in the three species. The remarkable conservation ranging from the genomic organization to the predicted amino acid sequences is indicative of the functional significance of N-cadherin molecular diversity.

Sequence inspection of the *N-cadherin*-neighboring region reveals the existence of six *N-cadherin-2* genes (designated *N-cadherin-2* through *N-cadherin-7*). The *N-cadherin-2* genes are more closely related to one another than to the *N-cadhein-1* gene. They likely arose from gene duplication events after *Drosophila* and *Anopheles* diverged. These *N-cadherin-2* paralogs and the *N-cadherin* ortholog span approximately 2 Mb (or about 0.8 percent) of the mosquito genome. In addition, the *N-cadherin-5* genomic sequence suggests that it might undergo alternative splicing as seen in the *N-cadherin*.

N-cadherin and LAR regulate R7 target at different stages

Yonekura, Lee

Fly vision is mediated by three classes of photoreceptor neurons (R-cells), each of which responds to a specific spectrum of light and connects to a specific layer in the brain. This wiring principle, named layer-specific targeting, is commonly observed in complex nervous systems, including vertebrate brains. The wiring pattern might facilitate information processing. We have chosen to study the connections made by the R7 class of photoreceptor neurons to the M6 layer in the medulla ganglion because those photoreceptors (1) are essential for visual behavior and (2) can be easily visualized by histology and manipulated by genetic means. Using the UV/VIS choice test, we previously identified five loci that affect R7 connectivity. These include *N-cadherin*, receptor phosphatase *LAR*, *milton*, and two novel loci. Mosaic analyses indicated that the genes function cell-autonomously in R7s (presynaptic termini), as removing any of them in R7 neurons results in targeting defects. Comparing the expressivity of different mutant phenotypes suggests that *N-cadherin* and *LAR* are the major determinants of R7 target selection.

To gain insight into the mechanism of action of N-cadherin and LAR, we performed developmental analysis coupled with high-resolution image analysis. We found that N-cadherin is required for initial targeting of R7 axons. Removing *N-cadherin* in single R7 axons results in approximately 15 percent of mutant R7 axons failing to project into the presumptive R7-recipient layer. While the mutant growth cones may reach the R8-recipient layer (or further into the intermediate layer) and extend their filopodia into the presumptive R7-recipient layer, the growth cones proper fail to move into the correct layer, as seen in the wild type. We propose that *N-cadherin* mutant R7 growth cones are able to sample but fail to recognize their targets. Alternatively, N-cadherin might be involved in translating target-derived signal into directional movement of the growth cone proper. By 25 percent pupal development, about 50 percent of R7 axons retract out of the R7-recpient layer, suggesting that N-cadherin plays a role in the maintenance of R7–target interaction. Furthermore, the *N-cadherin* mutant R7 growth cones exhibit various morphological defects. They fail to expand fully once they reach the correct layer, and some expand prematurely before reaching the target layer. We speculate that N-cadherin–mediated adhesion induces the remodeling of actin-based cytoskeleton, which, in turn, modulates growth cone morphology.

In contrast, the initial targeting of *LAR*-mutant R7 axons is normal. We observed no morphological defect in growth cones at the early stage. The *LAR*-mutant R7 growth cones remain in the R7-recipient layer up to 25 percent pupal development. We have previously shown that the *LAR*-mutant R7 axons retract at the later stage (at about 35 percent pupal development). Thus, LAR is required for stabilizing the interaction between R7 growth cones and their targets but not for initial targeting. Removing both N-cadherin and LAR in R7 axons results in the initial targeting defects seen in *N-cadherin* mutants. In addition, *N-cadherin* and *LAR* double mutant R7s show higher expressivity of targeting defects at adult stage than that of *N-cadherin* or *LAR* single mutants. Based on differential onset of mutant phenotype and the enhanced phenotype in the double mutants, we suggest that N-cadherin and LAR have distinct functions in R7 target selection.

We are currently analyzing the other loci that affect R7-dependent behavior. We found that three loci, including one known gene, *milton*, affect the morphology of R7 synaptic termini. Milton is associated with kinesin and its function is required for axonal transport of mitochondria to synapses. The role of mitochondria in neuronal target selection is not known and is a subject of future study. We are currently performing developmental analysis on *milton* and other mutants to understand their roles in R7 target selection.

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RETROTRANSPOSONS AS MODELS FOR THE REPLICATION OF RETROVIRUSES

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The medical importance of retroviruses such as HIV-1 has intensified the need to understand the molecular details of reverse transcription and integration. As a result of their similarity to retroviruses, long terminal repeat (LTR)-retrotransposons in simple eukaryotes are a powerful model, with information about LTR-retrotransposons possibly leading to the identification of new antiviral strategies or targets that could be used to combat the spread of HIV-1. The retrotransposon studied in our laboratory is the Tf1 element of the fission yeast *Schizosaccharomyces pombe*. One of our objectives is to map the residues of reverse transcriptase (RT) that mediate individual steps of reverse transcription. Another important objective of our research is to identify the mechanisms responsible for the insertion preference of Tf1 at pol II promoters. Our work is largely motivated by similar preferences that have been reported for the integration of HIV-1 and murine leukemia virus. Our third objective centers on the possibility that the nuclear import of viruses such as HIV-1 could be the target of antiviral therapies. We have chosen as an important goal the study of Nup124p, a nuclear pore factor that is specifically required for the import of Tf1.

The primer grip in the RNase H of RT, a conserved domain that contributes specifically to removal of the plus-strand primer from the 5′ end of the cDNA

Atwood-Moore, Ejebe, Levin; in collaboration with LeGrice

The reverse transcription of retroviruses and LTR-retrotransposons unfolds as a complex sequence of reactions that produce several critical intermediate products, including the initial minus-strand product of cDNA called a strong stop, the extended minus strand, the plus-strand primer (PPT), the plus-strand strong stop, and, ultimately, the full-length double-stranded cDNA. The synthesis of these intermediates requires both the DNA polymerization and RNase H activities of RT. The RNase H domain must degrade the RNA once it is used as template, recognize and preserve the PPT, and then remove the PPT after it has primed plus-strand synthesis. Although much is known about the amino acids that catalyze the DNA synthesis and RNA degradation, much less is known about the residues and structures required for the recognition and removal of the PPT.

To identify which residues of RT mediate the interactions with the PPT, we used random mutagenesis of Tf1 RT and screened for mutations that allowed the synthesis of cDNA intermediates but inhibited integration. We reasoned that the mutants would likely be defective in the late steps of reverse transcription such as those requiring interactions with the PPT. We generated a collection of 3,000 strains that were unable to support transposition and screened them with a genetic assay that detects intermediates of reverse transcription. We found 35 strains that, despite their defect in transposition, exhibited wild-type levels of homologous recombination. Immunoblot analysis demonstrated that the mutations did not affect levels of RT and integrase (IN). Surprisingly, DNA blots showed that many of the mutants produced what appeared to be normal levels of full-length, double-stranded cDNA. The remaining elements produced an interesting array of incomplete products.

Our experiments focused on a cluster of mutations in RNase H that included a region with five mutations in a five–amino acid segment. Interestingly, the transposons with mutations in this region produced normal levels of full-length double-stranded cDNA. We demonstrated the significance of this domain by a crystallographic study of HIV RT showing that the domain is a "primer grip" that interacts directly with the nucleotides of the PPT adjacent to the position cleaved by RNase H to create and then remove the plus-strand primer. Both the position and the sequence of the primer grip residues in HIV corresponded to the cluster of mutations we identified in the RNase H of Tf1. The specific residues corresponded to HIV RT residues 475, 497, 501, 502, 503, and 505. These observations led us to propose that the mutations in the RNase H of Tf1 that result in full-length cDNA are defective for transposition because the recognition of the PPT was altered. A defect of a few nucleotides in the cleavage of the PPT from the 5′ end of the plus strand would be "templated" into altered sequences at the 3′ ends of the minus strand, producing a drastic impact on the ability of IN to catalyze strand transfer.

To test the hypothesis that the cluster of mutants in RNase H altered the cleavages of the PPT, we characterized the sequences of the 3′ end of the minus strand cDNA. We used RNA ligase to attach an oligo to the 3′ ends of the cDNA to amplify by PCR a product containing the junction of the 3′ end. We inserted the product into a vector and 150 to 200 independent junctions and sequenced them for each mutant RT. In the 3′ ends of the cDNA produced by wild-type Tf1-*neo*, just 35 percent corresponded to the full-length species. However, the mutations showed a significant drop in the full-length species and correlated with an increase in 3′ ends that retained the PPT sequence. Thus, our screen for mutations in RT that make cDNA products identified the primer grip of RNase H as contributing to the removal of the PPT RNA from the end of the LTR. Surprisingly, this ability to process the PPT is specific and not required for the other steps in the pathway of reverse transcription.

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Tf1 encodes a Gag protein that is a functional equivalent of the Gag proteins of retroviruses

Teysset, Kim, Dang, Levin

To determine whether the 27 kDa protein encoded at the N-terminus of the Tf1 ORF is the functional equivalent of a retroviral Gag, we generated deletions of 10 amino acids in each of the four regions of hydrophylicity. We tested the transposition activities of Tf1 elements with the deletions, referred to as Tf1-delta-A, Tf1-delta-B, Tf1-delta-C, and Tf1-delta-D, by using a genetic assay that is based on the resistance to G418 resulting from the insertion of a *neo*-marked copy of Tf1. The transposition assays showed that Tf1-delta-B and Tf1-delta-C exhibited no transposition at all; Tf1-delta-A and Tf1-delta-D generated very low levels.

To test whether the deletions in Gag caused defects in the production of cDNA, we used DNA blot analysis, which showed that Tf1-delta-A generated a wild-type level of cDNA. Tf1-delta-D produced significantly lower levels of cDNA while repeated examination of Tf1-delta-B and Tf1-delta-C detected no cDNA bands. The data indicated that the reduced levels of transposition observed for Tf1-delta-B, Tf1-delta-C, and Tf1-delta-D were attributable to significantly reduced levels of reverse transcription. On the other hand, the reduced transposition caused by Tf1-delta-A likely reduced transport of Tf1 into the nucleus. We base our conclusion on our work that identified a nuclear localization signal within the sequence deleted by Tf1-delta-A. To investigate whether the transposition defects were attributable to decreased stability of Tf1 proteins, we performed immunoblot analysis with antibodies raised against the Tf1 Gag, IN, and RT. Although the deletions had little impact on the levels of RT and IN, Tf1-delta-B and Tf1-delta-C produced very low levels of Gag. Thus, the Gag protein was required for transposition.

Previous work established that Gag, RT, IN, and cDNA assemble into large macromolecular complexes that cosediment in sucrose gradients. We tested whether Tf1-assembled spherical virus-like particles (VLPs) can be observed with electron microscopy and found that particles with typical structures were easily detectable in the cytoplasm of cells expressing the Tf1-WT element. They appeared as clusters of well-defined particles or aggregates. The maximum diameter of these intracytoplasmic particles was 50 nm. We used the same techniques of electron microscopy to determine whether the forms of Tf1 with deletions in Gag produced spherical particles and found no particles or dense aggregates in the strains with Tf1-delta-C. Given that the Gag proteins were unstable in Tf1-delta-C, the results indicate that Gag was required for particle formation. In the case of Tf1-delta-A, we found dense aggregates in the cytoplasm and for Tf1-delta-D, with the size and number of particles similar to wild-type Tf1.

Many Gag proteins of retroviruses and LTR-retrotransposons are able to form particles in the absence of any other retroelement proteins. We tested whether the expression of the Tf1 Gag was sufficient for the formation of spherical particles. Tf1-PRfs, the version of Tf1 with the frame shift at the beginning of PR, does not express RT or IN. Electron microscopy revealed that a large percentage of cells expressing Tf1-PRfs contained particles and dense aggregates, indicating that Tf1 Gag is sufficient for the assembly of spherical particles.

One principal role of Gag proteins is to assemble into particles that package the RNA of the retroelement. We therefore tested whether Tf1 Gag was required for packaging the Tf1 RNA. Our approach followed a previously developed assay that detects the amount of Tf1 RNA protected from degradation by the nuclease Benzonase. The strains expressing Tf1-delta-B and Tf1-delta-C were unable to protect RNA from degradation, corresponding to the reduced levels of Gag and the lack of particles detected by electron microscopy. Thus, Gag was required for packaging the Tf1 mRNA.

In summary, the lack of transposition activity resulting from deletions B and C was attributable to the absence of Gag. The low levels of Gag resulted in no formation of particles, no packaging of mRNA, and no reverse transcription. The results of the electron microscopy demonstrated that the expression of Gag was sufficient for particle formation. Taken together, our results indicate that Tf1 encodes a Gag protein that is a functional equivalent of the Gag proteins of retroviruses.

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Retrotransposons and their recognition of pol II promoters: a comprehensive survey of the transposable elements derived from the complete genome sequence of *S. pombe*

Bowen, Levin; in collaboration with Epstein, Jordan, Wood

The integration of HIV-1 cDNA shows a significant preference for actively transcribed genes. Similarly, the insertion of murine leukemia virus shows a strong preference for sites within 5kb from where pol II initiates transcription. Very little is known about how these viruses interact with the structures of chromatin and recognize their target sites. The study of the retrotransposons Ty1 and Ty3 of *S. cerevisiae* has demonstrated clearly that much has been learned about integration directed to the promoters of pol III–transcribed genes. However, our recent observation that the integration of Tf1 occurs specifically at pol II promoters presents an opportunity to study in *S. pombe* an integration mechanism that parallels that of retroviruses.

The complete DNA sequence of the genome of *S. pombe* provides the opportunity to investigate the entire complement of transposable elements (TEs), their association with specific sequences, their chromosomal distribution, and their evolution. Through homology-based searches, we identified the sequences of Tf elements used in our analysis. Only two families of LTR retrotransposons, Tf1 and Tf2, are known to exist in *S. pombe.* They are closely related elements that differ only in their sequence of Gag and the LTRs. We used the nucleotide sequences of Tf1 and Tf2 to query BLAST against the entire genome of the laboratory strain of *S. pombe*, 972. Our analysis confirmed that there were no full-length Tf1 elements within the laboratory strain 972. There were, however, 13 full-length elements of Tf2. The full-length Tf2 elements are a homogeneous group with an average pairwise DNA sequence identity of 99.7 percent. The high level of sequence identity among the Tf2 elements indicates that the elements transposed very recently.

The solo LTRs found in the genome of *S. pombe* can be classified into at least three large groups as follows: (1) those that are closely related to Tf2; (2) those that are closely related to Tf1; and (3) many smaller families of LTRs that are more distantly related to Tf1 and Tf2. We derived these designations from a complete phylogenetic characterization of the LTRs by using DNA distance values from comparisons with LTRs of full-length Tf1 and Tf2 elements. The short distances of many of the terminal branches of Tf1 and Tf2 indicate that these retrotransposons represent the largest number of recently active elements within the genome.

To determine whether preferences for integration sites existed during the insertion of the 186 Tf sequences, we compared the locations of solo LTRs and full-length Tf2s with the positions of all 4,984 predicted open reading frames (ORFs) of *S. pombe.* We found that all insertions were located exclusively in intergenic regions of the genome, in intergenic spaces that contained pol II promoters. In addition, the LTRs were clustered within 300 nulceotides of the 5′ end of ORFs. These specific positions could be the result of selective pressures, either positive or negative, that favor populations of *S. pombe* with each of the patterns observed. Alternatively, the patterns of the Tf sequences that we observed could be strictly the result of biochemical mechanisms of integration. Each of the biases in the position of Tf sequences described above was very similar in pattern and magnitude to the positions of insertions resulting from the induction of Tf1 transposition under laboratory conditions. These extensive similarities argue strongly that the biases in the position of Tf sequences as observed in the genome of *S. pombe* result from biochemical preferences of integration for specific sites. The data indicate that Tf elements recognize and insert upstream of RNA polymerase II promoters.

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MOLECULAR MECHANISM OF FROG METAMORPHOSIS

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The laboratory is exploring molecular mechanisms in amphibian metamorphosis. The control of this developmental process by thyroid hormone (TH) offers a unique paradigm in which to study gene function in postembryonic organ development. During metamorphosis, different organs undergo vastly different changes. Some, like the tail, undergo complete resorption while others, such as the limb, are developed *de novo*. Most of the larval organs persist through metamorphosis but undergo dramatic remodeling in order to function in a frog. For example, tadpole intestine in *Xenopus laevis* is a simple tubular structure consisting of primarily a single layer of primary epithelial cells. During metamorphosis, it is transformed into a multiply folded adult epithelium, with elaborate connective tissue and muscles, through specific cell death and selective cell proliferation and differentiation. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis both *in vivo* by using transgenesis or hormone treatment of whole animals and *in vitro* in organ cultures offers an excellent opportunity to (1) study the developmental function of thyroid hormone receptors (TRs) and the underlying mechanisms *in vivo* and (2) identify and functionally characterize genes that are critical for postembryonic organ development in vertebrates.

Roles of cofactors in gene regulation by TR

Choi, Jones,[a] Paul, Sachs,[b] Tomita, Shi

TR regulates gene transcription by recruiting cofactors to target genes. In the presence of TH, TR can bind to coactivators while the unliganded TR binds to corepressors. Many biochemical and molecular studies have investigated such cofactors. Nonetheless, little is known about whether and how they participate in gene regulation by TR in different biological processes *in vivo*. Our focus is to investigate how TR uses different cofactors in the context of development in various organs. Frog metamorphosis is particularly well suited for such a purpose. Based on earlier studies, we have proposed a dual function model for TR during frog development; that is, the heterodimers between TR and RXR (9-*cis* retinoic acid receptor) activate gene expression during metamorphosis when TH is present while, in premetamorphic tadpoles, they repress gene expression to prevent metamorphosis, thus ensuring a tadpole growth period.

To investigate how TR activates gene transcription during metamorphosis, we have started to study p300 and SRC, which are among the best studied coactivators, *in vitro* and in cell culture. We have obtained the cDNA clones for *Xenopus* p300, SRC2, and SRC3 and have shown that they are expressed during metamorphosis. To investigate their involvement in gene regulation by TR, we used the frog oocyte system that we had established earlier for studying TR function in the context of chromatin. By using a chromatin immunoprecipitation (ChIP) assay, we recently demonstrated that TR can recruit SRC3 to a target promoter. Furthermore, we have generated a dominant negative form of SRC3 (dnSRC3) that can be recruited by TR, thus blocking the recruitment of wild-type SRC and gene activation. Currently, we are carrying out similar studies on p300. More important, we are introducing the dnSRC3 into tadpoles through transgenesis to investigate whether SRC recruitment is important for gene regulation by TR in developing animals and for frog metamorphosis.

To determine whether and how TR represses target genes in premetamorphic tadpoles in the absence of TH, we have cloned the full or partial coding regions of the corepressors N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors), which are the best-studied corepressors that bind to TR *in vitro*. We have shown that both are expressed and, more important, bound to TH-response genes in premetamorphic tadpoles. Their association with TH-response genes is reversed upon treatment of the tadpoles with TH, indicating that their binding to TH-response gene promoters is attributable to recruitment by unliganded TR. In agreement with the ability of these corepressors to form complexes containing histone deacetylases (HDACs), TH treatment leads to an increase in local histone acetylation at the TH-response genes at least in the intestine and tail, suggesting that histone acetylation is an important factor in gene regulation by TR.

In addition to HDACs and N-CoR or SMRT, corepressor complexes are known to contain other proteins, one of which is the TBLR1 (transducin beta-like protein 1–related protein). We have recently shown that the frog TBLR1 is expressed in the tadpoles and thus likely participates in gene repression by unliganded TR. To investigate this possibility, we again made use of the frog oocyte system to study promoter regulation in the context of chromatin. We have shown that endogenous N-CoR forms a complex with TBLR1 and that both N-CoR and TBLR1 can interact with unliganded TR *in vivo*. Using a ChIP assay, we have demonstrated that unliganded TR recruits TBLR1 as well as N-CoR to its target promoter, leading to histone deacetylation and gene repression in chromatin. Furthermore, using a dominant negative N-CoR that contains only the TBLR1-interacting domain, we showed that TBLR1 is required for transcription repression by unliganded TR. Finally, by comparing the function of TR, RAR, and the human leukemia-related fusion proteins PML-RARalpha and PLZF-RARalpha, we provided the first *in vivo* evidence for targeted recruitment of N-CoR-TBLR1 complexes by different types of nuclear receptors in transcriptional repression in the context of chromatin.

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Function of TR during development

Buchholz, Hsia, Shi

Our dual function model for TR during frog development argues that transcriptional activation by TR is essential for frog metamorphosis. To test such a hypothesis, we have adapted the sperm-mediated transgenic method to generate transgenic animals expressing a dominant negative TR (dnTR). Phenotypic analysis indicates that dnTR overexpression inhibits TH-induced metamorphosis.

More important, we have shown that dnTR specifically blocks the expression of TH response genes. To determine the underlying mechanism by which TR regulates TH response genes *in vivo*, we used a ChIP assay to show that dnTR is bound to target promoters, which leads to retention of corepressors N-CoR and SMRT and to continued histone deacetylation even in the presence of TH. The results thus provide direct *in vivo* evidence for a molecular mechanism by which a dnTR alters gene expression. The correlation between dnTR-mediated gene repression and inhibition of metamorphosis also supports a critical aspect of the dual function model for TR in development: during TH-induced metamorphosis, TR functions via the release of corepressors and promotion of histone acetylation to activate target gene expression, thereby inducing morphological changes.

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Involvement of matrix metalloproteinases during TH-induced tissue remodeling

Amano, Fu, Hasebe, Shi; in collaboration with Ishizuya-Oka

In an effort to identify genes important for postembryonic development, we isolated many TH response genes during metamorphosis. For functional investigations, expression analyses and other studies have led us to focus on the TH-response genes encoding matrix metalloproteinases (MMPs), extracellular enzymes capable of digesting various ECM components. Our earlier studies have led us to propose that the MMP stomelysin-3 (ST3) is directly or indirectly involved in ECM remodeling, which in turn influences cell behavior. Our work is supported by organ culture studies *in vitro,* in which we showed that ST3 function is important for TH-induced apoptosis of larval intestinal epithelial cells and for the invasion of the proliferating adult epithelial cells into the connective tissue. To investigate directly the roles of MMPs in developing animals, we are employing the transgenic approach to express wild-type and mutant MMPs in *Xenopus* embryos and tadpoles. By using a heat shock–inducible promoter to drive the expression of ST3 or an inactive mutant form of ST3, we generated transgenic tadpoles that express wild-type or mutant ST3 upon heat shock. Heat shock at tadpole stages led to overexpression of wild-type or mutant ST3 in all organs, although we observed no visible morphological changes of the tadpoles for up to four days. Analysis of the intestine showed that overexpression of wild-type but not mutant ST3 caused premature apoptosis in the tadpole epithelium, consistent with our earlier organ culture studies. Furthermore, the apoptosis is accompanied by drastic remodeling of the basal lamina or the ECM that separates the connective tissue and epithelium in the intestine. Taken together, our results suggest that ST3 directly or indirectly modifies the ECM, which in turn facilitates cell fate changes and tissue morphogenesis during metamorphosis.

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CONTROL OF CHROMOSOME TRANSMISSION FIDELITY BY THE SMC PROTEIN COMPLEXES

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The eukaryotic ATP-ases of SMC (structural maintenance of chromosomes) family form several essential eukaryotic protein complexes. Two of them, cohesin and condensin, are the focus of our studies. These complexes determine the higher-order chromosome structure and dynamics in eukaryotic cells. The condensin complex is the enzymatic activity driving chromosome condensation in mitosis and meiosis. The complex is thus the molecular machine of chromosome condensation, a process indispensable for proper individualization of chromatids and their separation during anaphase. Cohesin is a complex essential for establishment and maintenance of transient association between sister chromatids (sister chromatid cohesion, SCC), lasting from S-phase to anaphase. To understand the mechanisms of chromosome condensation and SCC and the molecular mechanics of cohesin and condensin, we use a combination of approaches to study the genetics, cell biology, and biochemistry of these complexes and their regulatory factors. The chromosome condensation studies focused on condensin regulation and the specificity of its targeting to the natural chromatin sites in budding yeast and human cells. In particular, we investigated the role of the epigenetic makeup of the genome and posttranslational modification pathways in condensin regulation . Our work on SCC centered around characterization of cohesin association with chromatin *in vitro*.

Condensin regulation and chromatin targeting

Ouspenski, Wang, Yong-Gonzales, Williams

In budding yeast and higher eukaryotes, condensin is composed of five essential subunits: Smc2, Smc4, Ycs5/Ycg1, Ycs4, and Brn1. Our previous work uncovered the essential role of condensin in individualization and compartmentalization of sister chromatids before anaphase. Currently, this role of condensin is believed to be fundamental to chromosome condensation while associated compaction of chromatin is thought to be a consequence. Despite substantial recent progress in understanding the molecular structure and enzymology of condensin, the mechanisms regulating its activity remain obscure. Our current condensin studies focus on mitotic targeting of condensin to chromatin. Our genetic studies established that several pathways determine proper condensin localization to the specific chromatin domains and thus regulate the chromosome condensation process. One such pathway is via the SUMO- (Smt3) modification machinery. Smt3 itself, Smt3-isopeptidase Smt4, and the E3 Smt3-ligases Siz1 and Siz2 play an important role in condensin targeting. We are investigating the molecular mechanism of such regulation. We found two condensin subunits to be modified in mitosis, both in yeast and mammalian cells, but the modifications were not dependent on Smt3-conjugation machinery. Major directions of current work are molecular analysis of posttranslational modifications of condensin, whole proteome screening for the essential targets of Smt3 conjugation *in vivo*, and, by using the purified SUMO-lation system, assessment of purified chromatin proteins as Smt3-conjugation targets *in vitro*.

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Mechanism of sister chromatid cohesion

Kagansky, Strunnikov

The protein machinery of SCC is extremely complex. It establishes and maintains the link between two sister chromatids from S-phase through anaphase. A critical player in cellular SCC activity is cohesin, an evolutionarily conserved four-subunit protein complex including essential proteins Smc1, Smc3, Scc1/Mcd1, and Scc3. As no enzymatic activity was found to be associated with cohesin *in vitro*, the molecular mechanism of cohesin action in SCC is unknown. To address this issue, we developed a purified *in vitro* SCC system, including a model chromatin template (dinucleosome) and recombinant cohesin complex. Analysis of condensin association with DNA and a defined chromatin probe demonstrated that the cohesin holocomplex forms a stoichiometric complex with chromatin. Binding of cohesin to chromatin was independent of linker DNA and histone tails. In contrast, cohesin was unable to bind to naked DNA (even derived from the natural cohesion sites). The properties of the cohesin-chromatin interaction agree well with a ring-like model of cohesin structure. The *in vitro* studies suggest that the cohesin ring is stabilized through interaction with nucleosomal cores. The current development of this experimental system, recapitulating an elementary sister chromatid cohesion reaction, focuses on complementation of the SCC reaction with additional proteins required for establishing and regulating SCC *in vivo*.

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STUDIES ON DNA REPLICATION, REPAIR, AND MUTAGENESIS IN EUKARYOTIC AND PROKARYOTIC CELLS

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Under optimal conditions, the fidelity of DNA replication is extremely high: it is estimated that, on average, only one error occurs in every 10^{10} bases replicated. However, as living organisms are continually subjected to a variety of endogenous and exogenous DNA-damaging agents, optimal conditions rarely occur *in vivo.* Even though all organisms have evolved elaborate repair pathways to deal with such damage, the pathways rarely operate with 100 percent efficiency; thus, the persisting DNA lesions are replicated, but with much lower fidelity than in undamaged DNA. Our project aims to understand the molecular mechanisms by which mutations are introduced into damaged DNA, a process commonly referred to as translesion DNA synthesis (TLS) or translesion replication (TR). The process is thought to be facilitated by one or more of the "Y-family" of DNA polymerases that are conserved from bacteria to humans. We have largely focused on *Escherichia coli*, primarily because, from a cellular, molecular, and genetic point of view, it is the best characterized. However, the past few years have witnessed remarkable progress in understanding TLS in higher organisms, including *Saccharomyces cerevisiae* and human cells. Thus, we have investigated TLS in all three kingdoms of life: bacteria, archaea, and eukaryotic cells.

Translesion replication in *Escherichia coli*

Valjavec-Gratian, Sentz, Chang, Woodgate; in collaboration with Goodman, Paetzel

In *E. coli*, efficient translesion replication of many DNA lesions occurs only when the UmuC protein physically interacts with a dimer of UmuD′ to form a heterotrimeric complex of $\text{UmuD}'_2\text{C}$ (known as *E. coli* pol V). Given that pol V is a low-fidelity enzyme, its activities within the cell are strictly controlled at multiple levels. For example, the *umu* genes are arranged in an operon and are negatively regulated at the transcriptional level by the SOS repressor, LexA. Despite the fact that the *umu* operon is one of the tightest regulated in the LexA-regulon, transcriptional regulation is incomplete, and the cellular levels of UmuD and UmuC are kept to a minimum, also through their rapid Lon-mediated proteolytic degradation. After cellular DNA damage, RecA protein nucleates on regions of single-stranded DNA and mediates the posttranslational self-cleavage of LexA, leading to its inactivation and the derepression of genes in the LexA-regulon, including *umuD* and *umuC.* Interestingly, UmuD undergoes a mechanistically similar self-cleavage reaction. In the case of UmuD, cleavage of its N-terminal 24 amino acids converts it to UmuD′ and activates it for its role in SOS mutagenesis. UmuD and UmuD′ both form homodimers but, when mixed, preferentially associate as an UmuD/D′ heterodimer, rendering UmuD′ susceptible to proteolysis by the ClpXP serine protease. Therefore, once cellular DNA damage has been repaired and the need for pol V has abated, degradation of the mutagenically active UmuD′ subunit helps return cells to a resting state.

In vitro replication assays reveal that regulation of the catalytic activity of pol V is also modulated through multiple protein-protein interactions. For example, pol V does not catalyze TLS alone but is instead an essential component of a multiprotein "mutasome" complex comprising RecA protein, beta sliding-clamp, and SSB. In collaboration with Myron Goodman, we investigated the *in vitro* stability of the polV (UmuD′$_2$C) complex and its ability to undergo subunit exchange with UmuD and UmuD′ proteins. As cellular DNA damage is repaired, the inducing signal declines, and the rate at which UmuD is converted to homodimeric UmuD′$_2$ subsides. Subunit exchange between the UmuD′$_2$C complex and intact UmuD has two dramatic effects; first, it most likely leads to the inactivation of UmuC's catalytic function as the protein aggregates as an insoluble precipitate; second, it results in the formation of a soluble UmuD/D′ complex in which UmuD′ is susceptible to rapid proteolysis by ClpXP. Subunit exchange between UmuD′$_2$C and UmuD may therefore provide *E. coli* with yet another mechanism by which it can curtail the activities of error-prone pol V.

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Structural analysis of lesion bypass

Boudsocq, Plosky, Woodgate; in collaboration with Yang

We previously identified and cloned a Y-family DNA polymerase from the thermostable archaeon *Sulfolobus solfataricus* P2, which we called DNA polymerase IV (Dpo4). In collaboration with Wei Yang, we solved, by X-ray diffraction, the crystal structure of Dpo4 in a ternary complex with an undamaged DNA template DNA and an incoming dexoyribose nucleoside triphosphate, revealing that, like all DNA polymerases, Dpo4 possesses a topology similar to a right hand with protein domains that resemble "fingers," a "palm," and a "thumb" as well as a unique domain called the "little finger," which helps the enzyme bind to DNA. Since the genetic identification 30 years ago of the Y-family polymerases, a number of a gain- or loss-of-function mutants have been isolated. Based on the structure of Dpo4, we attempted to explain the molecular basis for these phenotypes. It appears that some of the mutations are located in key structural residues while others affect the ability of the polymerase to bind to DNA or an incoming nucleotide; still others mapped to the surface of the protein and may possibly represent regions of contact between Y-family polymerases and their protein partners.

While Dpo4 possesses an overall topology similar to high-fidelity polymerases, the fingers, thumb, and palm domains are relatively stubby and the active site of the enzyme is large and exposed to solvent. We believe that the "cavernous" active site is large enough to accept bulky DNA adducts that normally block replication by high-fidelity polymerases whose active sites are more restrictive. We tested this hypothesis by determining two crystal structures of Dpo4 complexed with *cis-syn* thymine-thymine cyclobutane pyrimidine dimer (CPD)–containing DNA, in which the 3′ and 5′ thymine of the CPD separately serve as a templating base. As predicted, both bases of the covalently linked pyrimidine dimer are accommodated in the active site of Dpo4. The 3′ thymine of the CPD forms a Watson-Crick base pair with the incoming ddATP while the 5′ thymine forms a Hoogsteen base pair with the incoming ddATP in *syn* conformation. *In vivo*, UV-induced T→C and T→A mutations are observed at the 3′ thymine of a CPD due to misincorporation of dGMP or dTMP, yet mutations rarely occur at the 5′ thymine. When the 3′ T of the CPD serves as a template in Dpo4's active site, the close contact between the 5′ thymine and the polymerase induces a 3 Å shift of the CPD toward the major groove, thus leaving ample space for the 3′ T to form a wobble base pair with dGTP or dTTP, with each making two H-bonds. At the 5′ T of the CPD, however, the restraint imposed by the Hoogsteen base pair discriminates against guanine and pyrimidines, and only adenine, which makes two H-bonds with thymine either by Watson-Crick or Hoogsteen base pairing, is acceptable.

Dpo4 is relatively inefficient at replicating the 3′ thymine of a CPD compared with the distantly related eukaryotic pol eta, which replicates past CPDs as efficiently as undamaged DNA. Humans with defects in pol eta exhibit the *Xeroderma pigmentosum Variant* (XP-V) phenotype; afflicted individuals are extremely sensitive to sunlight, prone to cancer, and often die at a young age unless exposure to sunlight is minimized. To uncover the molecular basis for efficient bypass of CPDs by pol eta, we generated a model of pol eta complexed with a CPD and the *S. cerevisiae* pol eta structure. With the Dpo4-CPD structure held fixed, pol eta was brought in by superposition of its most conserved palm domain with that of Dpo4. Interestingly, the finger, thumb, and little finger domains of pol eta, which bear many similarities to the corresponding domains of Dpo4, apparently need to rotate 16°, 16°, and 45°, respectively, to be in position to interact with the DNA. In this modeled complex, pol eta undergoes a "closing" motion and makes numerous favorable contacts with the DNA. Structure-based sequence alignment suggests that several amino acid substitutions in human and yeast pol eta make the active site much less exposed to solvent than that of Dpo4. The solvent-occluded active site together with the conformational change from the "open" DNA-free to "closed" DNA-bound form may therefore facilitate pol eta's ability to bypass CPDs.

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In vitro characterization of human and murine DNA polymerase iota

Chumakov, McDonald, Mead, Vaisman, Woodgate; in collaboration with Hanaoka, Jerina, Kunkel, Ohmori, Wilson

Another polymerase that has been extensively characterized is human DNA polymerase iota (pol iota), which we identified. Pol iota is a Y-family DNA polymerase and a paralog of pol eta. Unlike pol eta, which bypasses UV-induced CPDs efficiently and accurately, the ability of pol iota to bypass CPDs varies, ranging from limited misinsertion opposite CPDs to complete lesion bypass. We hypothesized that such discrepancies derived from slightly different assay conditions and local sequence contexts surrounding each UV-photoproduct. Indeed, we found that pol iota can facilitate significant levels of unassisted highly error-prone bypass of a thymine-thymine CPD, particularly when the lesion is located in a 3′-A[T-T] A-5′ template sequence context and the reaction buffer contains no KCl. In contrast, when encountering a UV-induced 6-4 pyrimidine-pyrimidine (6-4PP) thymine dimer under the same assay conditions, pol iota inserts the correct base A opposite the 3′ T of the 6-4PP about a hundred times more efficiently and accurately than incorrect nucleotides while incorporation opposite the 5′ T is highly mutagenic. Our data suggest that, under certain conditions and sequence contexts, pol iota can bypass T-T CPDs unassisted and can efficiently incorporate one or more bases opposite a T-T 6-4PP. Such biochemical activities may therefore be of biological significance, especially in XP-V cells lacking the primary CPD bypassing enzyme, pol eta.

Pol iota is unique among the Y-family polymerases in that its fidelity is strictly dependent on template sequence. Most errors occur at template T, where the enzyme inserts three to 10-fold more dGMP than dAMP, while the fewest errors occur at template A, where misincorporations occur with a frequency of 1 in 10,000. Pol iota is also unique within the Y-family in that it possesses a deoxyribose phosphate (dRP) lyase activity. These combined activities suggest that the pol iota may participate in specialized forms of base excision repair (BER). In a collaborative study with Samuel Wilson and Thomas Kunkel, we mapped the domain structure of human pol iota by controlled proteolysis, which revealed that pol iota consists of a 48-kDa N-terminal domain and a protease-resistant 40-kDa "core domain" spanning residues Met79 to about 460, which contains the dRP lyase active site. To evaluate further the capacity of pol iota to participate in BER, we characterized the dRP lyase and DNA polymerase activities on DNA substrates representing BER intermediates. We found that pol iota was able to complement extracts derived from cells deficient in the well-characterized BER enzyme pol beta.

Based on phylogenetic relationships, the broad Y-family of DNA polymerases can be divided into various subfamilies consisting of UmuC (polV)-like; DinB (polIV/pol kappa)-like; Rev1-like; Rad30A (pol eta)-like; and Rad30B (pol iota)-like polymerases. In general, the *in vitro* properties of polymerases characterized within each subfamily are remarkably similar. An exception to this rule occurs with pol iota–like polymerases: the enzymatic properties of *Drosophila melanogaster* pol iota are more similar to those of *S. cerevisiae* and human pol eta than to those of the related human pol iota. We previously identified a murine homolog of pol iota and, in the past year, overproduced and purified the mouse enzyme. Biochemical characterization of murine pol iota revealed that its enzymatic properties are, in fact, similar to those of human pol iota in that it exhibits low-fidelity synthesis that is template sequence–specific. Thus, although pol iota most likely arose from a genetic duplication of pol eta as eukaryotes evolved, it would appear that pol iota from humans, mice, and possibly all mammals has been further subjected to evolutionary pressures that have "tailored" its enzymatic properties toward other function(s) specific for higher eukaryotes.

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In vivo characterization of human and murine DNA polymerase iota

McDonald, McLenigan, Frank, Plosky, Vidal, Woodgate; in collaboration with Gearhart, Hanaoka, Lehmann, Rogozin

Recent years have seen considerable progress in understanding the molecular mechanisms of low-fidelity DNA synthesis on undamaged and damaged DNA templates, yet little is known about the *in vivo* cellular functions/activities of the Y-family polymerases. Our previous collaborative studies with Alan Lehmann revealed that a recombinant fluorescent-tagged human pol iota is predominantly localized to the nucleus, where it associates with the cell's normal replication machinery. Following DNA damage, pol iota accumulates into discrete foci at sites of stalled replication forks. Interestingly, the pattern of foci formation is identical to that previously reported by Lehmann and colleagues for the related Y-family polymerase pol eta, suggesting that foci formation of damage-induced pol iota and pol eta is tightly coordinated within the cell. Indeed, we discovered that pol eta and pol iota interact with each other physically and that the C-terminal 224 amino acids of pol iota are sufficient for both the interaction with pol eta and its relocalization into replication foci. Our data therefore suggest that human pol eta and pol iota coexist in a larger holoenzyme complex, whereby their lesion-bypassing activities can be coordinated in response to DNA damage, and that both enzymes play a general role in maintaining genomic integrity and participating in translesion replication.

No disease has yet has been attributed to defects in human pol iota. In an effort to generate an animal model system in which to study the cellular function of pol iota, we set out to generate a targeted disruption of *Poli,* the murine gene encoding pol iota. First, we determined the entire genomic structure of the *Poli* gene located on a 120-kb mouse genomic BAC clone. Similar to human *POLI*, the mouse *Poli* gene has 10 exons spanning approximately 23 kb on mouse chromosome 18, band E2, which shares synteny with human chromosome 18. Sequence analysis led to the discovery that the BAC clone contained a single nucleotide polymorphism in exon 2 that changed the wild-type Ser-27 codon (TCG) to a TAG amber stop codon. Fortuitously, the mutation can be easily detected, as it destroys a *Taq*I restriction site in the wild-type gene. Using a PCR-based assay, we were able to genotype a variety of mouse strains for the presence or absence of the polymorphism, showing that 129/J, 129/SvJ, 129/ReJ, and 129/Ola mice are homozygous for the *Poli* nonsense mutation while C57BL/6J, BALB/c, and ICR Swiss mice have a wild-type *Poli* gene. Given that many of the roughly 148 genes involved in the cellular response to DNA damage have been knocked out in 129-derived embryonic stem cells, such findings are of interest to the DNA repair community because any such knock-out mouse strains or mouse embryonic fibroblast cell lines might carry the *Poli* amber mutation. Although a biological function for DNA polymerase iota has yet to be elucidated, it is possible that the lack of pol iota activity in mice mutant for other repair genes might conceivably influence the radiation and chemical sensitivities, DNA repair characteristics, mutagenic potential, and oncogenic susceptibility of the targeted mutant mice and their derived cell lines.

Based on its unique biochemical properties, it has been hypothesized that pol iota might participate in the somatic hypermutation of immunoglobulin variable genes. The availability of mice naturally deficient in pol iota gave us an opportunity to test this hypothesis. We identified mutations in the intron region downstream of rearranged V genes on the heavy chain locus from both 129/SvJ (*Poli* $^{-/-}$) and C57BL/6J (*Poli* $^{+/+}$) mice. The overall frequency of mutation was 2.5 percent mutations/bp for 129/SvJ clones and 0.5 percent mutations/bp for C57BL/6J clones. The types of base substitution in the two groups of clones were similar, and the percent of individual types of substitution was highly similar between the two groups. Thus, it appears that either pol iota does not participate in hypermutation of immunoglobulin genes or its role is nonessential and can be readily assumed by another low-fidelity polymerase.

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TISSUE BIOPHYSICS AND BIOMIMETICS

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We try to understand fundamental relationships between function and structure in soft tissues, in "engineered" tissue constructs, and in tissue analogs. Specifically, we study how the microstructure, hierarchical organization, composition, and material properties of tissue affect its normal biological function and dysfunction. We employ a physical perspective to address these questions, which entails using quantitative methods (e.g., physical measurements and mathematical models) to interrogate biological and physical model systems. Primarily, we use water to probe both equilibrium and dynamic interactions occurring among tissue constituents over a wide range of time and length scales. To determine the equilibrium osmo-mechanical properties of well-defined model systems, we systematically vary their water content and ionic composition. To probe tissue structure and dynamics, we employ small-angle x-ray and neutron scattering, static and dynamic light scattering, and magnetic resonance imaging (MRI). Conversely, we try to understand how essential transport processes (e.g., of mass, charge, momentum) are affected by changes in tissue microstructure. MRI methods have provided the most direct noninvasive means to characterize these transport processes *in vivo*; the methods have also been successful in characterizing normal tissue microstructure and in following changes in development, degeneration, and aging. Another goal of this section is to translate our quantitative experimental methodologies from "bench to bedside."

Imaging water diffusion in the brain and in other soft tissues

Pierpaoli, Basser, Jones, Jarisch; in collaboration with Aldroubi, Assaf, Barnett, Cohen, Freidlin, Marenco, Pajevic

We are continuing to develop water displacement imaging methods that allow us to probe different features of tissue microstructure and to assess and diagnose neurological and developmental disorders. Diffusion Tensor MRI (DT-MRI) is the most mature such technology. With it, we measure a diffusion tensor of water, D, on a pixel-by-pixel basis within tissue, both noninvasively and *in vivo*. Information derived from D includes local fiber-tract orientation, the mean-squared distance that water molecules diffuse in any given direction, the orientationally averaged mean diffusivity, and other intrinsic scalar invariant quantities that are independent of the laboratory coordinate system. These scalar parameters behave like quantitative histological or physiological "stains" that are "developed" without requiring exogenous contrast agents or dyes. We continue to find these quantitative parameters to be useful in characterizing tissue microstructure and its physiologic state. For example, the bulk or orientationally averaged diffusivity has been the most successful imaging parameter proposed to date to identify ischemic tissue regions in the brain during and following an acute stroke. Measures of diffusion anisotropy are effective in identifying white matter degeneration (Wallerian degeneration) associated with chronic stroke. DT-MRI also provides new information that can be used to follow early developmental changes occurring in cortical gray and white matter. Such changes cannot be detected with other imaging methods.

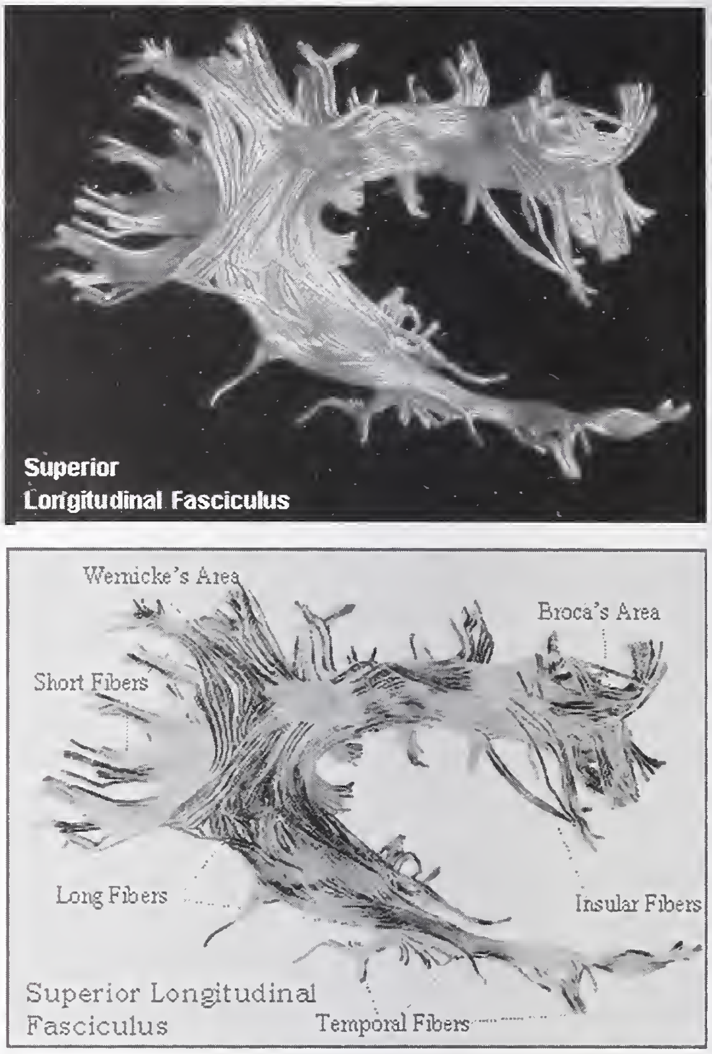

FIGURE 15.1

Rendering of the superior longitudinal fasciculus (SLF). DT-MRI data were obtained from a normal human subject. We then performed tractography from seed points chosen within the SLF. Juxtaposed is a line drawing showing well-known anatomical landmarks as they might appear in a classic neuroanatomy text. Adapted from: Catani M, Howard RJ, Pajevic S, Jones DK. Virtual *in vivo* interactive dissection of white matter fasciculi in the human brain. *Neuroimage* 2002;17:77-94.

Recently, we developed ways to use the water diffusion data to perform "*in vivo* virtual dissections" of the brain, i.e., to perform detailed studies of the brain's structural anatomy that previously could be performed only with laborious, invasive histological methods. One approach, pioneered by Sinisa Pajevic and Carlo Pierpaoli and independently by Derek Jones, entails encoding nerve fiber orientation by color. That approach has allowed us to identify the main association, projection, and commissural white matter pathways in the brain and even to differentiate white matter pathways of similar structure and composition but different spatial orientations. To assess anatomical connectivity between different functional brain regions, we recently proposed and demonstrated "fiber tractography," a way to trace out or track nerve fiber tract trajectories by continuously following the direction along which the apparent diffusivity is a maximum. Figure 15.1 shows an example of white matter pathways computed in living brain. While the reliability of DT-MRI tractography is high in the coherent primary white matter pathways, artifactual tracts can be produced by following smaller white matter bundles, for instance, where they cross, merge, branch, and so forth. By using complementary MRI data and more detailed models of water diffusion processes in tissue, we are developing approaches for classifying and correcting for these artifacts.

Clinical and biological applications of DT-MRI (and of other novel displacement MRI methods) have required the creation of a new mathematical, statistical, and image-sciences framework for analyzing these novel multidimensional data sets. Akram Aldroubi and Sinisa Pajevic developed algorithms for obtaining a continuous, smooth approximation to the discrete, noisy, measured diffusion tensor field data, thereby allowing us to reduce noise and enabling us to follow fibers more reliably. We have also derived the form of a new Gaussian distribution that governs the statistical variability of diffusion tensor data. This new parametric distribution has proved useful in establishing criteria for designing "optimal" DT-MRI experiments. We have developed nonparametric (bootstrap) methods for determining features of the data's statistical distribution from experimental DT-MRI data. The parametric and nonparametric statistical methods enable us to apply powerful statistical hypothesis tests to address a wide range of important biological and clinical questions that previously could be treated only by using *ad hoc* methods. More recently, in association with Yaniv Assaf and Raisa Freidlin, we have been developing new MRI sequences in conjunction with more detailed mathematical models of tissue microstructure and morphology. These models are enabling us to infer more microstructural information about white and gray matter than DT-MRI can currently provide.

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MRI study of normal brain development

Pierpaoli, Basser, Rohde, Chang, Carandang, Horkay

Many obstacles have impeded neuroimaging research on childhood brain disorders, including children's limited ability to comply with procedures, the lack of an adequate normative database with which to characterize normal brain development and against which to identify and measure aberrant brain development, the lack of adequate image analysis tools for characterizing developmental changes in the brain, and a lack of adequate means for sharing data analytic tools. Three NIH institutes (NICHD, NIMH, and NINDS) have jointly sponsored a multicenter study to learn more about how the brain develops in typical healthy children and adolescents. The study will enroll approximately 500 children, ranging from infancy to young adulthood, who will be seen over a six-year period at several clinical centers around the United States. The centers will perform MRIs of the brain and will relate the imaging findings to the results of standardized neuropsychological tests. In addition to facilitating the study of normal human brain development and providing a necessary, representative, and reliable source of normal control data for studies of children with brain disorders and diseases, the study will provide growth curves for normal brain anatomy and aid in the development of pediatric neuroimaging tools. More information regarding the project and our group's specific tasks can be found at the study Web site at *http://www.brain-child.org/*.

Our role is to serve as a data processing center, in particular to process and analyze all DT-MRI data that will be acquired by the various centers. To date, Gustavo Rohde has been developing methods to "warp" and register multidimensional DT-MRI data sets obtained from children. Lin-Chin Chang has been developing several Web-based applications that allow diffusion tensor data to be imported from the various centers and examined for quality control purposes. Ferenc Horkay is developing new polymeric phantoms that can be used to calibrate each DT-MRI measurement system in each center to ensure that the centers' DT-MRI acquisitions are quantitative and of sufficiently high quality.

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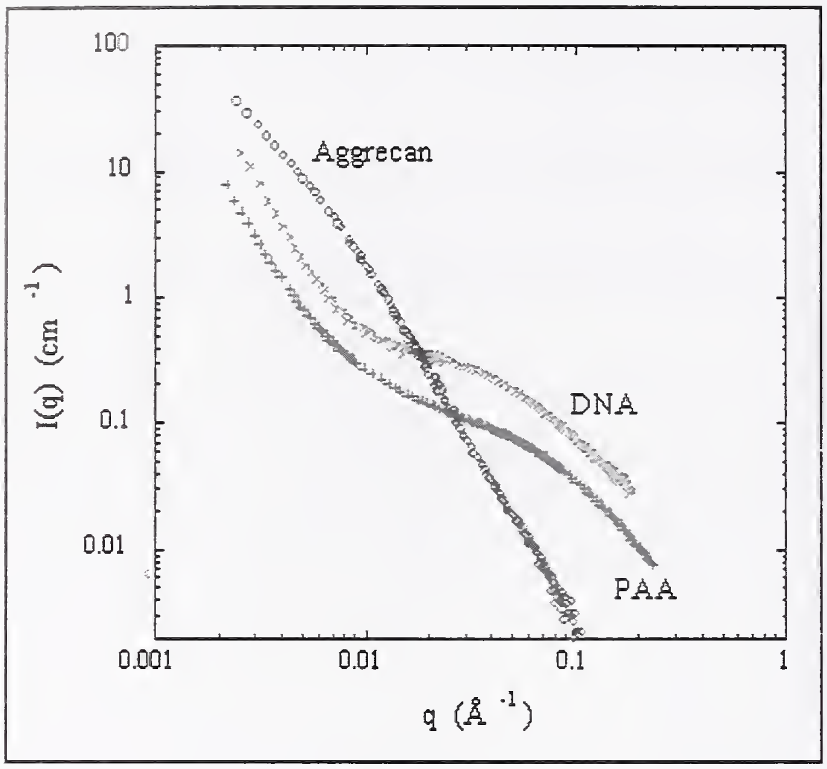

FIGURE 15.2

DNA and polyacrylate exhibit qualitatively similar behavior, which differs significantly from the matrix protein, aggrecan. Charged groups residing on the polymer backbone as well as mutual hydrophobic interactions introduce structural domains in the charged polymers that are not present in neutral polymers. In DNA, the double helix promotes a hierarchy of structures of different characteristic length scales, which depend sensitively on the DNA concentration, the ionic strength, and the chemical nature of the counter ions.

Water-ion-biopolymer interactions

Horkay, Basser; in collaboration with Geissler, Hecht

To help us understand the nature of physical/chemical interactions taking place in biomolecules and biomolecular assemblies, we have developed an approach for simultaneously studying structure (morphology) and thermodynamic properties as a function of the length scale (spatial resolution). The method combines macroscopic osmotic swelling pressure measurements and small-angle neutron scattering (SANS). Macroscopic swelling pressure measurements probe the system in the large-length scale range, thus providing information on the overall thermodynamic response. The SANS measurement simultaneously provides information about the size of different structural elements and their respective contribution to the osmotic properties. By combining measurements, we can separate the scattering intensity arising from thermodynamic concentration fluctuations from the intensity scattered by large static superstructures (e.g., aggregates) and determine the length scales relevant to the macroscopic thermodynamic properties. Such thermodynamic and structural information cannot be obtained by other techniques.

Specifically, we have applied our approach to the interactions of multivalent cations (e.g., calcium) with various biomolecules. Even though such ions are ubiquitous in the biological milieu, existing theories do not adequately explain the ions' effects on and interactions with charged macromolecules in general and biomolecules in particular. Moreover, experiments to study these interactions are difficult to perform, particularly in solution; multivalent cations cause charged molecules to phase-separate or precipitate above a low-concentration threshold. We have overcome this limitation by cross-linking our biopolymers. Given that macroscopic phase separation does not occur in cross-linked gels, we are able to extend the range of ion concentrations in which the system remains stable. We have applied our method to pilot studies of cross-linked gels of polyacrylic acid, a model synthetic polymer, and of DNA. Concentrated DNA solutions and DNA gels have never before been investigated by using SANS in conjunction with osmotic measurements (see Figure 15.2). The proposed method also provides a unique framework for analyzing the osmotic and scattering behavior of other biomolecular systems.

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Functional properties of extracellular matrix

Horkay, Basser; in collaboration with Washburn

Knowledge of cartilage hydration (water uptake) is essential to understanding and predicting its load bearing and lubrication properties since it is primarily the osmotic properties of cartilage that cause it to resist applied compressive loads. Cartilage swelling behavior is also exquisitely sensitive to changes occurring in development, disease, degeneration, and aging. Moreover, controlled hydration or swelling of cartilage also provides a means of determining functional properties of cartilage and of other extracellular matrices.

We previously introduced the concept that the controlled hydration of cartilage could also be used to measure important physical/chemical properties of the collagen network and proteoglycans (PG) in cartilage independently within the extracellular matrix. This approach entailed modeling the cartilage tissue matrix as a composite material consisting of two distinct phases: a collagen network and a proteoglycan solution trapped within it; applying various known levels of equilibrium osmotic stress; and using physical-chemical principles and additional experiments to determine a "pressure-volume" relationship for both the PG and collagen phases independently. In pilot studies, we used this approach to determine pressure-volume curves for the collagen network and the PG phases in native and trypsin-treated normal human cartilage specimens, as well as in cartilage specimen from osteoarthritic (OA) joints. In both normal and trypsin-treated specimens, collagen network stiffness appeared unchanged, whereas in the OA specimen, collagen network stiffness decreased. Our findings highlight the role of the collagen network in limiting normal cartilage hydration and ensuring a high PG concentration in the matrix, which are essential for both effective load bearing in cartilage and lubrication, but of are lost in OA. These data also suggest that the loss of collagen network stiffness, and not the loss or modification of PGs may be the incipient event leading to the subsequent disintegration of cartilage observed in OA.

One shortcoming of this approach, however, was that it required many days to study a single cartilage specimen, so the approach was not suitable for routine pathological analysis or tissue engineering applications. Secondly, a significant amount of the tissue specimen, not routinely available in most clinical and biological applications, was required to obtain the osmotic titration curves. This contributed to long equilibration times in obtaining data. Therefore, we have recently developed a new micro-osmometer to perform these experiments in a practical and rapid manner. This instrument can measure minute amounts of water absorbed by small tissue samples (less than 1 microgram) as a function of the equilibrium activity (pressure) of the surrounding water vapor. A quartz crystal detects the water uptake of a specimen attached to the crystal's surface. The high sensitivity of its resonance frequency to small changes in the amount of adsorbed water allows us to measure the mass uptake of the tissue specimen precisely. Varying the equilibrium vapor pressure surrounding the specimen induces controlled changes in the osmotic pressure of the tissue layer. To illustrate the applicability of the new apparatus, we recently measured the swelling pressure of tissue-engineered cartilage specimen.

The micro-osmometer will eventually permit us to obtain a profile of the osmotic compressibility or stiffness of multiple cartilage specimens simultaneously as a function of depth from the articular surface to the bone interface. The instrument will also allow us to quantify the contributions of individual components of extracellular matrix (ECM), such as aggrecan and hyaluronic acid, to the total osmotic pressure, and should allow us to assess the osmotic compatibility and mechanical integrity of developing tissues and of tissue-engineered cartilage (or ECM) for implantation.

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Physical-chemical aspects of cell and tissue excitability

Horkay, Basser; in collaboration with Hallett, Miranda, Tasaki

The primary goal of this work is to explain key features of cell and tissue excitability, many aspects of which are still poorly understood from a physical/chemical perspective. We have observed several anomalous phenomena that we have shown to be necessary to elicit an excitatory response, including volume and temperature changes in the superficial protoplasmic layer of nerve axons, which coincide with the action potential waveform. These changes coincide with a first-order phase transition in nerve cells, fibers, and synapses caused by the exchange of divalent cations, such as calcium, with monovalent cations, such as sodium and potassium. Our previous experiments with perfused axons implicate divalent/monovalent cation exchange as a mechanism by which nerve fibers can be excited in an "all-or-none" manner. To understand the physical-chemical basis of these temperature and volumetric changes, particularly how divalent/monovalent cation exchange can induce such changes, we are studying these processes in synthetic "biomimetic" anionic polymer gels under quasi-physiological solution conditions. An advantage of studying the behavior of these gel model systems is that, unlike living tissue, we can control their structure, composition, and interactions among their components. Thus, in synthetic polyacrylate gels, Ferenc Horkay has observed that minute changes in the concentration of divalent cations in the surrounding liquid can induce significant changes in chain stiffness in the gel, even if ion binding is weak and completely reversible. Various physical-chemical and polymer physics–based techniques, including neutron scattering, osmotic swelling, and mechanical loading, provide complementary information about these biologically relevant phenomena over a wide range of length scales. These basic studies are leading to a deeper understanding of the physical mechanisms underlying nerve excitation.

We are also investigating biophysical aspects of stimulation of tissue in the central and peripheral nervous systems by electromagnetic induction (magnetic stimulation). Using the finite element method, Pedro Miranda has performed detailed calculations to predict the electric field and current density distributions induced in the brain during magnetic stimulation. We have found that both tissue heterogeneity and anisotropy of the electrical conductivity contribute significantly to distort the induced fields, and even to create excitatory or inhibitory „hot spots" in some regions. This new understanding could affect the interpretation of clinical findings, and improve our understanding of how nerves are excited electromagnetically in different brain regions.

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We develop new biophysical and optical methodologies for biomedical research and clinical applications. Of late, we have been focusing on the refinement of technological approaches to characterizing early stages of disease and to developing strategies for prevention of progression and for monitoring responses to therapy in cancer and age-related macular degeneration (AMD). In further developments of Laser Capture Microdissection and related laser technologies, we are seeking to optimize the isolation of specific populations of cells from sections of complex tissues with global analysis of specific molecular changes. We have applied statistical analysis of gene expression to identify candidate lists for stage-specific molecular markers within microdissected cell populations, focusing on technical improvements necessary to identify critical but less abundantly expressed genes, proteins, and lipids.

Laser microdissection technology development

Bonner; in collaboration with Emmert-Buck, Mage, Obiakor, Pohida

As the list of expressed human genes is completed, a major scientific and medical challenge will be to track the complex molecular events that drive normal tissue differentiation and progression of pathologic lesions. With refinements in PCR, hybridization microarrays, and mutation screening, both DNA and mRNA can be extracted from tissue biopsies or cytology specimens and analyzed with a parallel panel of hundreds or even thousands of genetic markers. Given that cells in complex tissue are biochemically and physically affected by surrounding cells as well as by remote stimuli from greater distances, the task of analyzing critical gene expression, protein, and lipid patterns in development, normal function, and disease progression depends on the extraction of specific cells from their complex tissue milieu. In collaboration with NCI, our laboratory invented and developed Laser Capture Microdissection (LCM) (Arcturus Engineering, Inc., has commercialized the technology). We have also tried to evaluate and improve alternative commercial laser microdissection technology (using pulsed UV laser microablation). A variety of tissue preparation and microextraction protocols allow LCM to isolate pure populations of specified cells both rapidly and reliably from microscopic regions of tissue sections in a form useful for subsequent quantitative, multiplex molecular analysis using PCR and RT-PCR amplification and microarray analysis. The laser microdissection techniques are now widely used in molecular analysis of genetics and gene expression changes within target cells in complex tissues. However, in global proteomic and lipid studies without molecular amplification methods, the quantity of isolated cells that is sufficient to perform accurate characterization of less abundant species is problematic as the microscopic visualization, targeting, and isolation in laser microdissection has a maximal rate of about 1 to 20 cells per second depending on the cells' microscopic distribution within the tissues.

In parallel efforts, we have been investigating the biophysics of UV laser ablation and have demonstrated the role played by polymer breakdown and ionization in electrostatic interactions determining target collection efficiency. Polymer multilayer coatings with charge neutralization appear to improve ablative laser microdissection, thus offering particular advantages for collecting larger populations of cells required for automated cytometry and many proteomics discovery efforts. In collaboration with NIAID, we have used nested PCR and DNA sequencing to demonstrate reliable molecular analysis in LCM-isolated immunolabeled single cells at the single-molecule level. As we proceed to specific cell isolation and greater knowledge of molecular changes in pathology, specific molecular targeting will become increasingly important to ensure rapid, precise collection of subpopulations that are not discernible by morphology alone.

In an effort to lead technology integration with biological and clinical research, we have studied the polymer physics and chemistry of the thermoplastic microtransfer processes. Recent improvements include "noncontact LCM" and methods that use automated beam steering and pulse sequences. The LCM process, which loads picoliter cell volumes onto equivalent volumes of polymer matrix, is being applied to integrated purification and analysis of specific macromolecular constituents. We have been able to emboss a patterned microfluidic circuit into the LCM polymer tapes and directly load selected cells into a specified well of approximately 1 nanoliter volume.

Recently, in collaboration with the Emmert-Buck laboratory and CIT, we invented and are now refining an automatic microtransfer technique based on molecule-specific labeling of cells not requiring user visualization or microscopic targeting and capable of much higher throughput rates. This technique (patent pending) is built on our solid physical understanding of thermoplastic microtransfer and uses a much simpler device than commercial laser microdissection instruments. Our current prototype is capable of isolating all specifically immunolabeled cells within a 1 cm^2 region of a standard immunostained tissue section in about five seconds, which corresponds to specific separation from approximately 50,000 cells per second. With this technique we can exceed the cell separation rates of standard technologies such as fluorescence-activated cell sorting while preserving our ability to harvest directly from standard sections of complex tissues as well as maintaining spatial location within the tissue. As this technology becomes more robust, we will seek to integrate it with molecular profiling of specific cells within tissues, including routine proteomic and lipidomic analyses, particularly for the large number of less abundant molecular species (lower copy number per cell).

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Gene expression complexity and statistics of gene expression

Kuznetsov, Bonner; in collaboration with Emmert-Buck, Mulshine

If microdissection and molecular analysis can be made clinically practical, the expression levels of sets of approximately 20 to 100 critical, stage-specific disease markers within a selected cell population might provide reliable diagnosis and intermediate endpoints of response to molecular therapies in individual patients. Our analysis of large gene expression databases (SAGE and LCM cDNA libraries) suggests that a significant fraction of all genes is expressed in any specific cell type and that the levels of gene products universally exhibit a highly skewed power-law distribution similar to those characterizing many other complex systems. We have developed mathematical models for the evolution of such distributions that predict the observed distributions of genes, protein domains, and gene expression observed in species of increasing biological complexity.

To follow macromolecular changes during cancer progression within prostate, breast, lung, and ovary tissues, we have developed, in collaboration with NCI, standard procedures for the isolation of normal and pathological cells from clinical specimens. Using statistical multivariate analysis, we have developed mathematical algorithms for the selection of "candidate genes" from cDNA libraries. The candidate genes' expression frequencies are strongly correlated with disease progression. We use our models of the statistics of expression levels in cell populations to identify genes differentially expressed in cancer progression. To date, our analysis points to a critical role for many less abundantly expressed genes at a critical stage of ovarian cancer progression and suggests that, for most cancers, critical diagnostic marker sets should include such low-abundance transcripts. This notion is guiding our research in statistics of less abundant gene products and suitable detection methods.

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Gene expression during normal development and pathology progression

Parlato,[a] Malekafzali,[b] Bonner; in collaboration with DiLauro, Mushinski

We have applied single-cell LCM capture technology to studies of gene expression during normal development (spermatogenesis and thyroid bud development) and in stage-specific pathological cells (e.g., cancer precursors). In collaboration with Roberto DiLauro's laboratory, we examined gene expression patterns associated with the primordial thyroid and the adjacent cells from which it derives in both wild-type and knock-out mice. In collaboration with Fred Mushinski, we are applying the same LCM methodology to study early changes in the well-established plasmacytoma model in BALB/c mice. Jointly, we have developed robust LCM techniques for isolating specific cells from mouse cryosections and have recovered good yields of high-quality mRNA. In collaboration with Life Technologies, Inc., we have made cDNA libraries of high diversity and message length from 100 ng of microdissected mRNA (about 5,000 cells). Using Affymetrix microarray hybridization, we can determine global gene expression profiles in these libraries while preserving the libraries for isolation of the full-length message of identified stage-specific genes. We are planning to apply these methods, which have undergone validation in reproducible animal models, to clinical specimens of early stages of disease progression in which microdissection of critical rare cell populations is required. We foresee an evolution of molecular diagnosis from one based on the qualitative or quantitative analysis of a few key macromolecules to one in which special clustering algorithms analyze complex multivariate databases. Such analyses should permit a more complete identification of highly correlated clinical cases and allow us to characterize their response to molecular therapies specifically designed to prevent progression. To this end, we have refined the LCM of rare cell populations, particularly those that might be accessible by serial, minimally invasive cell harvesting from patients.

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Prevention of progression of age-related macular degeneration through photoprotection

Meyers, Ostrovsky, Ettori, Mahadi, Bonner

Age-related macular degeneration (AMD) is the most common cause of severe visual loss in the elderly. Early stages of the disease are ophthalmoscopically detected with increasing frequency with age (particularly over 60 years). Lipofuscin accumulates with age in the retinal pigment epithelium (RPE). It is the dominant fluorphore in the retina, and, in Stargardt's disease, its increased rate of accumulation is associated with early macular degeneration. This finding led us to hypothesize in the early 1980s that lipofuscin is photochemically active and responsible for acute retinal damage at high blue light levels, causing chronic photochemical (free radical production) stress that increases with age (lipofuscin accumulation). If lipofuscin photochemistry plays such a critical role in initiation and progression of early AMD, then simple optical spectral filters (e.g., short wavelength blocking and rod-sparing sunglasses) might be expected to reduce dramatically the rate of adverse photochemistry in the outer retina while optimally preserving normal photopic vision in moderate to bright ambient light. We designed such filters (Bonner: 500-nm-long pass sunglasses; Ostrovsky: 430-nm-long pass intraocular lens) that have been used clinically. The recent discovery of a photochemical active product (A2E) created by rod photoreception at high light levels and our experimental determination of A2E photochemical action spectrum in RPE cell culture led us to reinvestigate optimal designs for optical filters to reduce or prevent progression of early AMD. We have generated models of spectral irradiance in the human macular RPE in which irradiance progressively decreases as the lens ages but dramatically increases in critical blue wavelengths following cataract surgery and intraocular lens (IOL) implantation. The models suggest the importance of the reduction of both the maximal bleaching of rod rhodopsin and the rate of singlet oxygen generation from phototoxic photoproducts (trans-retinal, A2E, and its photoproducts), along with the significance of the role of photodegradation of A2E and its toxic photoproducts by visible light; the models also predict dramatic increases in adverse photochemistry following cataract surgery without additional photoprotection. We have proposed spectrally selective "sunglasses" for reducing both rod activation in bright ambient light and the accumulation and activation of toxic byproducts of photo-transduction. In addition, we are seeking to characterize spectrally different RPE photoproducts to evaluate the potential for noninvasive monitoring of the molecular effects of such filters in patients. In collaboration with Sergei Avetisov and his colleagues at the Eye Institute of the Russian Academy of Sciences, we are designing clinical studies of the effects of such filters on progression of pre-existing AMD following cataract surgery and IOL implantation.

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APPLICATIONS OF PHOTON MIGRATION TO TISSUE TOMOGRAPHY AND SPECTROSCOPY

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Our goal is to devise new approaches for noninvasive, quantitative optical spectroscopic and tomographic imaging of deep tissue structures for clinical screening and monitoring of physiological parameters. To achieve this goal, we have undertaken a multifaceted theoretical, computational, experimental, and clinical research program that includes time-resolved transillumination of thick tissue applied to quantitative spectroscopy of breast tumors; studies of the use of specific fluorescent markers (e.g., ligands) for identifying the molecular biology of disease processes applied to noninvasive biopsy of Sjögren's syndrome; and lymphatic imaging for sentinel node detection. To bring these methodologies from bench to bedside, we are using rodents as test cases for our quantitative imaging concepts. We are involved in several clinical studies, including an NCI protocol to use oblique angle reflectometry for noninvasive monitoring of inflammation in the oral cavity, an NIDCR/NINDS clinical study to evaluate the drug response of patients experiencing complex regional pain syndrome, and another NCI-sponsored clinical trial to evaluate the effectiveness of anti-angiogenesis drug treatment for Kaposi's sarcoma. We have started to model the stochastic process of tumor-induced angiogenesis in the extracellular matrix.

Time-resolved tomography of thick tissue

Chernomordik, Hattery, Gandjbakhche; in collaboration with Cubbedu, Rinneberg, Weiss, Zaccanti

We continued our joint project with the Physikalisch-Technische Bundesanstalt of Berlin and Politecnico di Milano aimed at quantifying optical properties of breast abnormalities (e.g., tumors) by using time-domain scanning mammographs designed by the Berlin and Milan groups. Our analysis of their multispectral *in vivo* data, obtained at several projections, is based on the random-walk model of time-resolved contrast functions. It results in estimates of the blood volume and oxygen saturation of the tumors and surrounding tissues. In most cases, we were also able to reconstruct sizes of the abnormalities. Preliminary findings on the small sample of patients indicate that the tumors (invasive ductal carcinoma) are hypoxic and have increased blood volume as compared with surrounding normal tissue. We have started a joint project with the Canadian company Advanced Research Technology to install its time-resolved imaging system at NIH. We will use the analysis of the *in vivo* data obtained with the device to characterize tumors and study the correlation between the spectroscopic signatures of the breast tumors and the risk associated with the outcome of therapy in several clinical protocols. Using Monte Carlo simulations and phantom experiments, we are also continuing to expand and substantiate our analysis of photon migration inside turbid tissues, especially for decreased scattering abnormalities that require special treatment, as some tumors as well as cysts do show decreased scattering. We have devised a theory that accounts for different diffusion coefficients for structured tissue such as white matter of the brain or muscle. In this formalism, photons entering the tissue will diffuse mostly in the direction of the fibers rather than in the perpendicular directions. We will design special phantoms to test the theory.

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Three-dimensional reconstruction of localized *in vivo* fluorescence

Chernomordik, Hattery, I. Gannot, Gandjbakhche; in collaboration with G. Gannot, Russo, Smith

The development of specific fluorescently labeled cell surface markers has opened the possibility of specific and quantitative noninvasive diagnosis of tissue changes. We are pursuing the development of a fluorescence scanning imaging system that can perform a "noninvasive optical biopsy" of Sjøgren's syndrome (SS) and replace the currently used histological biopsy. In our animal experiments, we have devised an imaging system to quantify the concentration of fluorescinated antibodies binding to B or T tumor cells previously injected in the oral cavity of mice. Preliminary results show that our imaging system, along with our theoretical algorithm, is able to monitor noninvasively the concentration of fluorophore signals as well as the pharmacokinetics associated with wash-out of the antibodies. We are also continuing our study of infrared-dependent fluorescent detection methods to determine the position of sentinel lymph node(s) to replace currently used detection by radioactive particles. We have extended our analysis of different phantom data and *ex vivo* tissue to confirm the potential of the random walk–based theoretical approach to reconstruct with good accuracy three-dimensional positions of deeply embedded fluorophores. In a series of animal experiments, we were able to use near-infrared nanoparticles to study the uptake of the fluorophores through the blood circulation and in tumors. We were able to image these dynamics in real time.

We are also investigating whether our model of diffuse fluorescent photon migration is able to separate the effects of light diffusion at a given depth from the actual distributions of the fluorescent antibodies. In one experiment, we first injected the tongues of Balb-C mice with squamous carcinoma cells that are CD3- and CD19-positive and then injected the tongues with FITCI conjugated antibodies to CD3 or CD19. To assess the pharmacokinetics of the fluorescent antibodies, we imaged the tongues at different time intervals after injection of the fluorescent antibodies. In a separate experiment, we used a similar procedure except that we covered the tongues with slabs of agarose gel, whose properties mimic those of human tissue. We used two pertinent parameters, i.e., change in peak intensities of the fluorescent signals and their full width at the half maximum (FWHM) as a function of time, to study the dynamics of labeled antibodies. As expected, the results of the two experiments exhibited different functional forms as a consequence of the "degeneration" of the signal by tissue scattering. Using our model of diffuse fluorescent photon migration, we are able to "deconvolve" the signal coming from deep structures and retrieve the true pharmacokinetics of tumor cells. The results show clearly that our mathematical model is able to quantify not only the concentration of fluorescently labeled antibodies but also their clearance and diffusion through tissue.

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Oblique angle reflectometry for noninvasive monitoring of inflammation: application to chemopreventive drugs

Hattery, Hekmat, Gandjbakhche; in collaboration with Mulshine

Inflammatory cell populations produce cytokines that can specifically stimulate growth of evolving cancer clones. Given that normal epithelium shares many biological properties with cancer cells, it also responds to the chronic presence of mitogenically active cytokines with accelerated growth (hyperplasia); normal cell hyperplasia provides a measure of the promotional environment of a cancer clone. At NCI, a Phase II trial is under way to determine the effectiveness of a cyclooxygenase (COX) inhibitor on oral leukoplakia. The effectiveness will be monitored by surgical biopsy with its associated morbidity. As a less morbid approach to monitoring patient response, we have developed a noninvasive quantitative epithelial inflammation-measuring device that may be used to evaluate the general state of the oral mucosa and monitor the effectiveness of chemopreventive treatment regimes. The device measures diffuse reflectance from a low-power optical wavelength source, which inserts photons at specific angles into the tissue. As the angle of insertion approaches parallel with the epithelial surface, the mean penetration of the photons becomes smaller and the photons spend more time in the epithelial layer. Wavelengths of interest are those with high tissue absorption, thus limiting detected photons to those with few scattering events and confining to the surface layers the tissue volume that has been interrogated. From a theoretical point of view, this approach permits the use of integral equations to describe photon migration in a two-layer tissue model. We performed several phantom experiments by using acrylamide gel with high absorption covered with a thin liquid layer to simulate the epithelial layer of tissue and compared the data with the theoretical predictions. Using our theoretical model, we collected and analyzed 10 sets of data from five patients and two controls. Initial results indicate that the model is effective in indicating the level of inflammation in patients. An improved device using flexible imaging fiber bundles is also under construction. In this new design, an imaging bundle replaces a set of optical fibers. Instead of obtaining output readings from each fiber sequentially, a CCD camera will record light distribution instantaneously, promising shorter acquisition time and higher spatial resolution. We have run high-resolution Monte Carlo simulations on a high-performance computing cluster for comparison with data obtained from phantoms. Analysis of the data indicates that epithelial thickness may be quantified with an uncertainty of approximately 30 percent, even if patient variations in scattering are as high as a factor of four and variations in absorption are as high as a factor of 10 Thus, the new technique should be particularly robust as well as clinically relevant. A company is currently evaluating the technology for licensing.

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Blood circulation in Kaposi's sarcoma and complex regional pain syndrome type I

Hassan, Hattery, Hekmat, Vogel, Byram, Gandjbakhche; in collaboration with Dionne, Yarchoan

We are investigating three imaging modalities to quantify different parameters associated with blood circulation. In the first case, thermography provides a two-dimensional image of superficial skin temperatures. The concept is that higher temperatures occur in the skin superficial to veins that are involved in active transport of blood. In the second case, laser Doppler imaging (LDI) produces two-dimensional images of blood flow over a defined area at 690nm and 780nm. In the third case, multispectral imaging, designed in our unit, produces two-dimensional images at six wavelengths (700 to 1000nm) by using a differential absorption algorithm (devised by our unit). The multispectral imaging technique permits determination of parameters related to the oxy-/deoxy-hemoglobin ratio (i.e., oxygenation) and total blood content as well as of changes in other metabolic and disease-related compounds such as cytochromes and ferritins.

Two studies using the techniques are now under way. An NCI-sponsored clinical trial is evaluating the effectiveness of anti-angiogenetic drug treatment in Kaposi's sarcoma (KS), a highly vascular tumor, in whose development and progression angiogenesis and capillary permeability can play an important role. Responses to KS therapy are now generally assessed visually by measuring and palpating the numerous lesions and using complex response criteria; no noninvasive standard technique is available to assess the effect of anti-angiogenetic therapy on blood flow. The purpose of the clinical trial is to test three noninvasive methods to assess of vascularity and the vascular changes associated with KS. For 20 patients, we recorded thermography, LDI, and multispectral images over the lesion for comparison with normal skin either adjacent to the lesion or on the contralateral side both before therapy and then after administration of a regimen of liposomal doxorubicin and interleukin-12 for 18 weeks. Comparative image analysis between LDI and thermography showed a strong correlation between results obtained with the two techniques. Measured by LDI, the KS lesions generally exhibited increased temperature and blood flux compared with normal skin. Interestingly, we observed that few patients had lower temperature in the lesion than normal. The multispectral data show a large increase in blood volume as well as intriguing patterns of cytochrome concentration changes within the tumor. These patterns may yield information on the detailed effects of drugs on specific tumors. Our techniques are objective, easy to perform, and appear to be highly sensitive in assessing improvement in the lesions upon administration of therapy; thus, they may have utility in monitoring trials of anti-angiogenesis therapy in KS patients.

Although complex regional pain syndrome type I (CRPS-I), formerly known as reflex sympathetic dystrophy (RSD), has long been recognized clinically, its pathogenesis is not understood. We are investigating the sensitivity and specificity of infrared thermography and LDI for tracking physiological parameters associated with CRPS-I before, during, and up to five weeks after therapy. Subjects are referred with a diagnosis of CRPS in one limb only. All subjects received an experimental drug or placebo for five weeks. All treatments were double-blinded. For six patients, we compared thermograms of the painful areas with adjacent and nonpainful contralateral regions to determine the differential temperature pattern. We acquired the LDI images consecutively with thermographic imaging. The thermal pattern on the painful side before drug or placebo treatment was warmer or cooler by at least 1°C and spread over a large area while no remarkable difference in LDI blood velocity images was observed between the two sides. By applying cold stimulation to an extremity for 10 seconds, the temperature dropped suddenly on both sides and increased over time; thermal recovery time was longer on the painful side than the pain-free side. After the drug treatments, thermography showed smaller temperature differences between pain and pain-free sides and an improved thermal recovery time. To date, the sample size is too small to draw firm conclusions. However, the results suggest that thermography can be used to locate and track the response to treatment of neuropathic dysfunction as the cause of chronic pain.

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Stochastic modeling of tumor-induced angiogenesis in a heterogeneous medium: the extracellular matrix

Amyot, Pourzand, Gandjbakhche; in collaboration with Camphausen

Angiogenesis plays an essential role in the establishment of malignant tumors. Tumors stimulate angiogenesis by releasing tumor angiogenic factors (TAF), such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). In response to these angiogenic stimuli, endothelial cells need to degrade the extracellular matrix (ECM), migrate through the extracellular matrix, proliferate, and organize themselves into a patent blood vessel.

The ECM constituents play an important role during both angiogenesis and vascular morphogenesis, and its cytoskeleton gives support to the cell, allows it to grow, and helps self-organization. During endothelial cell (EC) migration through the ECM, the cells secrete adhesive proteins that permit the ECs to adhere to each other and to the ECM meshwork. This adhesion aids in the construction and extension of new vessels, which organize around the meshwork and guide the formation of the future vascular network.

We have started a program to model tumor-induced angiogenesis. Our stochastic model begins with the secretion of TAF from cells of a solid tumor. The TAF molecules diffuse in the ECM until the endothelial cells absorb them. In response to the TAF, recruitment and cell migration initially power sprout formation and elongation. Sprout movement is a chemotactic response to the tumor-induced TAF gradient; the locomotion of the sprout is governed by the directionality of the endothelial cells that constitute the cell tip. Additional sprout extension takes place when cell division occurs in a narrow region juxtaposed to the inactive sprout tip. The vascular network generated in numerical simulations is similar to networks observed experimentally. Our model tests the influence of the distribution of ECM material on the formation of the vascular network. We have calculated the vascular density for different ECM network densities and have found that ECM heterogeneity has a barrier effect on angiogenesis: below a given value of heterogeneity, the sprouts cannot perfuse the tumor and a vascular network cannot form. Our simulation results indicate that the ECM network function is similar to a percolation medium between the tumor-secreting TAF and the vascular sprout. Depending on the ECM heterogeneity, a threshold for perfusion, comparable to the classical value of an ECM percolation threshold, exists, which allows the tumor to become vascularized. In collaboration with our colleagues at NCI, we are devising cell culture experiments to relate our theoretical finding to quantifiable angiogenesis processes.

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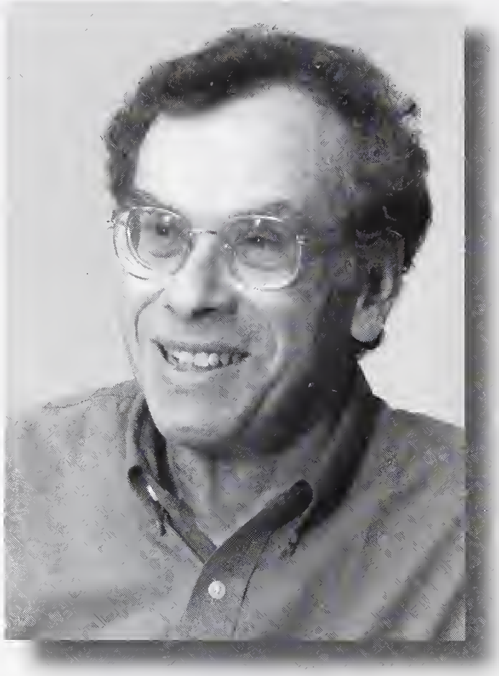
CELL BIOPHYSICS



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We are studying various elements of cell processes involved in signal transduction, protein trafficking, and cell motility. We focus on understanding the interactions and transformations of supramolecular cellular assemblages such as protein-coated endocytic vesicles, metabolic signaling complexes, and cytoskeletal structures and networks. We are particularly interested in the way cellular activities are coordinated in space and time, and we develop and apply novel methodologies based on mathematical and physical principles. We are constructing specialized fluorescence-based optical instrumentation and use advanced electromagnetic scattering techniques that allow examination of structures and dynamics on nanoscopic length scales. We also develop mathematical models of specific aspects of supramolecular and cellular behavior.

Mechanics of coated vesicle formation

Nossal, Ferguson, Boukari, Sackett; in collaboration with Jin, Lafer, Muthukumar

Protein and lipid trafficking in eukaryotic cells involves small tubulovesicular entities whose generation from cell membranes is mediated by the binding of specific coat proteins. In earlier studies undertaken to understand the physical mechanisms by which such structures are generated, we developed methods to determine the mechanical properties of clathrin cages, the major structural elements of the protein coats of certain endocytic vesicles. We also investigated how intermolecular forces influence the critical concentration for polymerization, i.e., the concentration below which cages will not form. By comparing theory with existing data, we recently investigated the mechanical role of assembly proteins (APs) in mediating the *in vitro* formation of clathrin baskets. We demonstrated that the binding of APs to baskets prevents the legs of neighboring triskelions from sliding with respect to each other when the legs intertwine to form the struts of a completed cage. In addition, our analysis confirms that coats containing only clathrin and APs are unlikely to bend a typical plasma membrane into small vesicles whose size is similar to that of clathrin-coated vesicles, although special regions of the membrane might be amenable to transformation. In a related investigation, we have used dynamic light scattering (DLS) and fluorescence correlation spectroscopy (FCS) to study various aspects of basket assembly, discerning, for example, that triskelions in solution have an intrinsic pucker whose geometric form is close to that previously inferred by cryoelectron microscopic examination of intact baskets. DLS measurements indicate only a weak dependence of pucker on pH and other solution conditions. The results support the notion that clathrin triskelia have an intrinsic curvature and that the triskelia, when inserted into cages and coats, change their shape to conform to the geometry of the coat at the cost of relatively low strain energy.

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Mathematical analysis of cyclic metabolic pathways for phosphoinositide synthesis

Skupsky, Nossal

Considerable evidence indicates that phosphatidylinositide (PI) metabolism is involved in many cell-physiological events, including vesicle biogenesis, motility, and chemotaxis. The feedback and feed-forward character of cyclic pathways of PI metabolism suggest that the pathways may act as biochemical switches for such events. Using mathematical and computational methods, we have examined a biochemical scheme for regulation of PI signaling that includes the actions of PI kinases and phosphatases, phospholipase C, small g-proteins, and phosphatidic acid production. We have developed a mathematical model of eukaryotic gradient sensing that can be applied to understanding chemotactic behavior in immune and amoeboid cells. The basic phenomenology captured by the theory includes transient response to a uniform stimulus, persistent and amplified cellular polarization in the direction of a static gradient, and the ability to follow a changing gradient. The model is based on reaction diffusion equations that describe PI regulation in response to spatio-temporal patterns of ligand-mediated receptor activation. We have investigated four variants of the model that differ in the amount of nonlinearity included in positive feedback and in the degree to which points on the plasma membrane must compete for species that translocate from the cytosol. Depending on how these elements are included, qualitatively different cellular responses result. We have performed simulations based on the above-mentioned phenomenology and have designed experiments to determine which might best describe a given cellular system. To gain insight into endocytic vesicle formation, we have modified forms of the model by, for example, including coupling of membrane activities to membrane curvature.

Structure of tubulin polymers

Sackett, Nossal, Boukari, Galanis; in collaboration with Bates, Chernomordik, Fojo, Krueger, Losert, Werbovetz

Tubulin polymers are centrally involved in various critical cell functions, including mitosis, intracellular transport, establishment and maintenance of cell morphology, and cell motility. In such processes, tubulin appears prominently as microtubules; the disturbance of microtubules usually results in dramatic modification of cell behavior. However, tubulin polymers appear in many forms, dependent on environmental factors such as pH, salt concentrations, temperature, and the presence of small organic molecules that mediate tubulin-tubulin interactions. For some time, we have been investigating how these factors affect resultant supramolecular tubulin structure. In particular, we have undertaken studies aimed at establishing how certain marine natural products, including cryptophycin and dolastatin, induce single-walled tubulin rings. For example, by combining data obtained by sedimentation velocity and DLS measurements with images obtained by scanning transmission- and cryo-electron microscopy, we inferred that there are two well-defined, repeating bends that create the necessary curvature to cause such ring structures to form. Recently, we have used FCS to study the stability of the rings at low tubulin concentration (see below). In collaboration with other investigators and with the goal of identifying drugs active against *Leishmania donovani* and related organisms, we have also studied the ability of certain herbicide derivatives to target kinetoplastid parasite tubulin selectively. We also have initiated several studies of biomimetic systems aimed at understanding physical aspects of the self-organization of microtubules into large-scale arrays.

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Fluorescence correlation spectroscopy and fourier imaging (scattering) techniques

Boukari, Nossal, Michelman, Sackett; in collaboration with Bansil, Chernomordik, Krueger, Lafer, Schuck, Szu

We have been developing FCS as a tool to examine the structure and stability of supramolecular biological assemblies, with emphasis on closed polymeric forms such as tubulin rings, clathrin baskets, and virus capsids. Unusual assemblies resulting from the interaction of tubulin with a group of antimitotic marine natural products, including cryptophycin and dolastatin, have served as prototypes for applying FCS to such macromolecular entities. When formed in the presence cryptophycin, the peptide inhibits tubulin with a geometric diameter of 27 nm; dolastatin inhibits tubulin with a diameter of 44 nm. By using FCS to measure the hydrodynamic diameter and the apparent number of fluorescent entities as the samples are diluted, we have been able to monitor the stability of the rings at tubulin concentrations as low as 1 nM. We recently began studies using this methodology to examine the *in vitro* assembly of clathrin cages. We have also been using FCS, in conjunction with analytic ultracentrifugation, to study the structure and stability of conjugated protein-polysaccharide vaccines when diluted to low physiological concentrations not appropriate for interrogation by other physical techniques. In addition, we have undertaken related FCS investigations to study the transport of biological macromolecules through complex polymer networks and are now employing small-angle light scattering (SALS) to understand structural changes that occur when polyelectrolyte gels are subjected to electric fields. The latter involves mathematical modeling of the migration and redistribution of ions within the gel network in order to calculate the pH gradients that seem to be responsible for field-induced shape changes in the gels.

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Our research is directed at understanding the cellular and genetic events that regulate T lymphocyte development. Current studies focus on the role of T lymphocyte signal-transducing molecules in immature T lymphocyte (thymocyte) selection, a process essential for formation of the mature T cell repertoire. To analyze the function of specific signal-transducing proteins in T lymphocyte development, we use transgenic and gene targeting methods to create overexpression, dominant negative, and loss-of-function mutants in mice. In addition, we are employing molecular genetic techniques to identify and characterize the function of novel genes that are expressed in T lymphocytes.

T cell antigen receptor signaling in thymocyte development

Frolova, El-Khoury, Love; in collaboration with Shores

A major focus of our research has been the investigation of the role of T cell antigen reciptor (TCR) signal transduction in thymocyte development. Signal transduction sequences (termed Immunoreceptor Tyrosine-based Activation Motifs; ITAMs) are contained within four distinct subunits of the multimeric TCR complex (zeta, CD3-gamma, -delta, -epsilon). Di-tyrosine residues within ITAMs are phosphorylated upon TCR engagement and function to recruit signaling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell activation cascade. Though conserved, ITAM sequences are nonidentical, raising the possibility that the diverse developmental and functional responses controlled by the TCR may be regulated, in part, by distinct ITAMs. To determine if TCR signal-transducing subunits perform distinct or analogous functions in development, we generated zeta-deficient and CD3-epsilon–deficient mice by gene targeting, genetically reconstituted the mice with transgenes encoding wild-type or signaling-deficient (ITAM-mutant) forms of zeta and CD3-epsilon, and then characterized the developmental and functional consequences of the alterations on TCR signaling. The results of our studies demonstrated that TCR-ITAMs are functionally equivalent but act in concert to amplify TCR signals. We found TCR signal amplification to be critical for thymocyte selection, the process by which potentially useful immature T cells are instructed to survive and differentiate further (positive selection). We also found that potentially auto-reactive cells that may cause auto-immune disease are deleted in the thymus (negative selection). Thus, the multisubunit structure of the TCR may have evolved to enable complex organisms to develop a broad, self-restricted yet auto-tolerant T cell repertoire.

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Mechanism of CD5-mediated TCR signal inhibition

Park, Love

The cell surface protein CD5 negatively regulates TCR signaling and thus participates in thymocyte selection. Examination of CD5 expression during T cell development revealed that surface levels of CD5 are regulated by TCR signal intensity as well as by the affinity of the TCR for self-peptide ligands in the thymus that mediate selection. To determine if the ability to regulate CD5 expression is important for thymocyte selection, we generated transgenic mice that constitutively express high levels of CD5 throughout development. Overexpression of CD5 significantly impaired positive selection of some thymocytes (those that would normally express low levels of CD5) but not of others (those that would normally express high levels of CD5). These findings support a role for CD5 in modulating TCR signal transduction and thereby influencing the outcome of thymocyte selection. The ability of individual thymocytes to regulate CD5 expression represents a mechanism for "fine tuning" the TCR signaling response during development. Our results indicate that this potential for signal modulation may be particularly useful for generating the maximum possible diversity in the mature T cell repertoire. Given that the activation-induced binding of regulatory molecule(s) to sequences within the CD5 cytoplasmic domain is a probable mechanism for CD5 function, we generated transgenic mice that express a tail-less form of CD5 (mCD5) and then used both the intact and mCD5 transgenes to reconstitute CD5 surface expression in $CD5^{-/-}$ mice. The experiments revealed a critical function for the cytoplasmic domain in CD5 signaling. In an effort to determine how CD5 regulates signal transduction by the TCR, the laboratory is currently attempting to identify molecules that interact with CD5.

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Role of LAT in T cell development

Park, Love; in collaboration with Samelson, Shores, Sommers

Linker for Activation of T cells (LAT) is an integral membrane protein that functions as a critical adaptor linking the TCR to multiple downstream signaling pathways required for T cell activation. The distal four tyrosines in LAT (tyr^{136}, tyr^{175}, tyr^{195}, tyr^{235}) are necessary and sufficient for LAT activity in T cells, which includes activation of the calcium and MAP kinase (MAPK) downstream signaling pathways. These signaling pathways are also activated by a large number of other receptors and are required for the development and function of many cell types. Thus, their inactivation in all cells would likely result in embryonic lethality. However, by mutating specific LAT tyrosines, we have been able to uncouple the TCR from downstream signaling pathways in T cells without affecting the ability of other receptors or cells to use these pathways. We generated "knock-in" mutant mice that express LAT proteins containing single or multiple tyrosine-phenylalanine mutations of the four critical tyrosine residues. Knock-in mice that express the wild-type version of the protein exhibited normal T cell development, thereby validating the targeting strategy. Inactivation of all four distal LAT tyrosines yielded a null phenotype (identical to the LAT knock-out), demonstrating the critical role of the residues for T cell development. Surprisingly, knock-in mutation of the first tyr residue (tyr^{136}) resulted in a fatal lymphoproliferative disorder characterized by expansion and multitissue infiltration of $CD4^{+}$ T cells. Consistent with previous data demonstrating that tyr^{136} preferentially binds to phospholipase C-gamma, examination of the signaling response of T cells from these mice revealed a severe defect in TCR-induced/phospholipase C-gamma–mediated calcium flux. However, MAPK signaling was intact in these cells, indicating that the TCR was selectively uncoupled from the calcium but not from the MAPK pathway. The results reveal a critical role for LAT in coordinating downstream signals initiated by TCR engagement and demonstrate that this function is essential for normal T cell homeostasis. Current studies are using the LAT tyr[136] knock-in mice to determine the role of calcium signaling in thymocyte selection and are analyzing other LAT tyr knock-in mutants generated in our laboratory.

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Structure and signaling potential of the gamma/delta TCR complex

Hayes, Love; in collaboration with Fowlkes, Laky

Most vertebrate species contain two separate lineages of T cells that are distinguished by the antigen-binding clonotype-specific chains contained within their TCRs: alpha–/beta–T cells and gamma–/delta–T cells. Although the more abundant alpha/beta TCR has been extensively characterized, much less is known about the structure or function of the gamma/delta TCR, which is expressed on the smaller subset of gamma-delta T cells. We found that the subunit composition of the gamma/delta TCR differs from that of the alpha/beta TCR in that a component of the alpha/beta TCR, the CD3delta chain, is not present in gamma/delta TCRs, revealing a major difference in the subunit structure of the alpha/beta and gamma/delta TCRs. Interestingly, signal transduction by the gamma/delta TCR was found to be superior to that of the alpha/beta TCR as assessed by several criteria. Our data suggest that the structural difference between alpha/beta and gamma/delta TCRs may influence the signaling potential of the TCR complex and could have important functional consequences on T cell activation. Current studies involve further analysis of the effect of TCR subunit structure on signaling responses and aim to determine if TCR subunit composition influences T cell development and T cell lineage commitment.

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Role of the chemokine receptor CCR9 in T cell development

Uehara, Love; in collaboration with Farber

T cell development continues into adulthood and requires the periodic migration of T-progenitor cells from the bone marrow to the thymus. The ordered progression of thymocytes through distinct stages of development is also associated with migration into and between different thymus microenvironments where they are exposed to different growth factors and signals. Chemokines are a group of small, structurally related molecules that regulate trafficking of leukocytes through interactions with a subset of seven-transmembrane, G protein–coupled receptors. The chemokine CCL25 is highly expressed in the thymus and small intestine, the two known sites of T lymphopoesis. CCR9, the receptor for CCL25, is expressed on the majority of thymocytes, raising the possibility that CCR9 and its ligand may play an important role in thymocyte development. To investigate the role of CCR9 during lymphocyte development, we generated CCR9-deficient ($CCR9^{-/-}$) and CCR9-transgenic mice. Surprisingly, both T cell and B cell development appeared normal in $CCR9^{-/-}$ mice. However, bone marrow transplantation experiments demonstrated that lymphocyte progenitors from $CCR9^{-/-}$ mice had a markedly reduced capacity to repopulate the thymus when forced to compete with progenitor cells from $CCR9^{-/-}$ mice. In other experiments, overexpression of CCR9 in transgenic mice inhibited early thymocyte development and blocked the normal migration of immature thymocytes within the thymus. The results indicate that CCR9 participates in regulating both the migration of progenitor cells to the thymus and the migration of developing thymocytes within the thymus. However, CCR9 is not essential for normal T cell development, suggesting possible functional redundancy. We are currently testing this hypothesis by generating mice deficient in both CCR9 and CXCR4, a second chemokine receptor highly expressed on developing thymocytes.

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Exploring the function of developmental transcription factors in T cell development

Li, Love

Lhx genes encode a conserved family of proteins that function as transcription factors during embryonic development (see report by Heiner Westphal on page 244). Although these genes have been shown to play critical roles in the development of various organ systems, their possible role in lymphopoiesis has not been systematically examined. In addition, the Wnt signaling pathway has recently been found to contribute to T cell maturation, suggesting that Wnt proteins, as well as their receptors and inhibitors, are likely to have important functions during thymocyte development. We have initiated an RT-PCR–based screening for expression of these genes in fetal and adult lymphoid tissues. An attractive feature of the study is that knock-out mice and embryonic stem (ES) cells are already available for many of these genes. Thus, if the expression pattern of specific genes suggests a role in lymphopoiesis, we can rapidly extend our results by analyzing the lymphoid phenotype of knock-out mice or, in the case of embryonic lethality, generating chimeric mice using knock-out ES cells to study lymphopoiesis specifically.

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MOLECULAR GENETICS OF AN IMPRINTED GENE CLUSTER ON MOUSE DISTAL CHROMOSOME 7

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Genomic imprinting is an unusual form of gene regulation in which expression of an allele is restricted based on its parental origin. Imprinted genes are not randomly scattered throughout the chromosome but rather are localized in discrete clusters. One cluster of imprinted genes is located on the distal end of mouse chromosome 7. The syntenic region in humans (11p15.5) is highly conserved in gene organization and in expression patterns. Mutations disrupting the normal patterns of imprinting at the human locus are associated with Beckwith Wiedemann syndrome, a developmental disorder exhibiting many types of tumor. In addition, inherited cardiac arrhythmia is associated with mutations in the maternal-specific *Kcnq1* gene. Our unit uses mouse models to address the molecular basis for allele-specific expression in the cluster. In our studies, we hope to use imprinting as a tool with which to understand fundamental features of epigenetic regulation of gene expression. We are also using the mouse system to generate animal models for the several inherited disorders associated with the cluster. We have generated models to study defects in cardiac repolarization associated with loss-of-function mutations at *Kcnq1* and to understand specifically the effect of beta-adrenergic–mediated stress on the cardiac phenotype.

Molecular basis for allele-specific expression of the mouse H19 and Igf2 genes

Park, Jeong, Sellars, Pfeifer

Our studies on the mechanisms of genomic imprinting focus on the *H19* and *Igf2* genes, which lie at one end of the distal 7 imprinted cluster. Paternally expressed *Igf2* lies about 70 kb upstream of the maternal-specific *H19* gene. Using cell culture systems as well as transgene and knockout experiments *in vivo*, we have identified the enhancer elements responsible for activation of these two genes. They are largely shared and located downstream of the *H19* gene. Parent-of-origin–specific expression of both genes depends on a shared element (called the *H19DMR*) located just upstream of the *H19* promoter and thus positioned between the *Igf2* gene and the shared enhancers. The CpG sequences within this element are methylated specifically on the paternally inherited chromosome. Our conditional ablation of the element *in vivo* demonstrates that the nonmethylated *H19DMR* (i.e., the copy on the maternal chromosome) is continually required for silencing of the maternal *Igf2* allele. Knock-in experiments demonstrate that the *H19DMR* contains a methylation-sensitive transcriptional insulator. Thus, on the nonmethylated maternal chromosome, the active insulator within the *H19DMR* prevents activation of *Igf2* by the downsteam enhancers. Methylation of the paternal chromosome inactivates the insulator and permits *Igf2* expression. Unexplained by this model is the effect of several small *DMR*s proximal to the *Igf2* transcription unit. Current studies are directed at the mechanistic significance of these elements. Imprinting of *H19* occurs via a distinct genetic mechanism. The conditional ablation of the *H19DMR* indicates that it is not continuously required to silence the paternal allele. Rather, the *H19DMR* is required early in development to establish an epigenetic state at the *H19* promoter that itself prevents transcription. Current studies indicate that the epigenetic program includes, but is not restricted to, the hypermethylation of the *H19* promoter.

To determine the elements that are necessary and sufficient for imprinting at the locus, we have moved the *H19DMR* and its mutated derivatives to heterologous loci. Our results demonstrate that the *DMR* alone is sufficient to imprint a normally nonimprinted chromosome. Moreover, this activity does not depend on germline differences in *DMR* methylation. Thus, the *DMR* likely marks its parental origin by a mechanism independent of DNA methylation. With genetic and molecular analyses of embryonic stem cells derived from mutant mice, we are now determining the epigenetic signals that constitute the genomic imprint.

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Mouse models for inherited long QT syndrome

Casimiro, Pfeifer; in collaboration with Ebert, Knollman

Inherited long QT syndrome (LQTS), which can result in syncope or sudden death, is characterized by an abnormal electrocardiogram indicative of repolarization defects. Romano-Ward syndrome (RWS) patients inherit the LQTS disorder generally as a dominant phenotype and show no other traits. Jervell and Lange-Nielsen syndrome (JLNS) patients display profound congenital deafness in addition to LQTS, with both phenotypes recessive. We have generated several mutations in the mouse *Kcnq1* gene to model the human diseases. Ablation of the gene results in vestibular and auditory defects. Histological analyses suggest that the defects are due to deficiency in the K^+-recycling pathway that is crucial for generating endolymph, the specialized fluid bathing the inner hair cells. ECG tracings of mutant mice indicate profound defects in cardiac repolarization when measured *in vivo*. However, the defects are not noted in isolated hearts *ex vivo*, indicating that the Kcnq1 protein plays a pivotal role in mediating critical extracardiac signals. Further anlayses demonstrate that Kcnq1 function is specifically required to modulate cardiac function in the presence of beta-adrenergic stimulation.

We have also generated three point mutations to model RWS. We have analyzed mutations in the central pore region and in the sixth membrane-spanning domain of Kcnq1. The phenotypes of these mutations are each a distinct subset of those seen in the null mutation and thus demonstrate that the Kcnq1 protein plays distinct roles in the heart versus the inner ear and in various aspects of cardiac function. While inherited LQTS is relatively rare, the genetic models represent excellent paradigms for addressing mechanisms for acquired LQTS, the single largest cause of death in Western societies.

Biochemical and pharmacological studies both predicted that the key biological role of the Kcnq1 protein was its association with the helper protein, Kcne1, to form the I_{KS} potassium channel. One of the most novel results of our studies is the discovery that that ablation of the *Kcnq1* gene leads to cardiac defects in addition to those noted in *Kcne1*-deficient mice. The results suggest a novel role for Kcnq1 in heart development and/or function. We have used our mutant mice as tools to detect a previously unappreciated potassium channel that was dependent on Kcnq1 but not on Kcne1. The role of this channel in mouse and human hearts is now under investigation.

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Beta-adrenergic hormone–synthesizing cells and development of the cardiac conduction system

Boe, Pfeifer

During early development, the heart is the primary (and probably the only) site of synthesis of the beta-adrenergic hormones norepinephrine and epinephrine. This cardiac-specific synthesis is transient and disappears by late gestation. Intriguingly, the cells synthesizing the beta-adrenergic hormones are located in positions that predict the location of the developing cardiac conduction network. To understand the fate of cells that synthesize these hormones, we generated a mouse with a mutated *Pnmt* locus such that that cre recombinase enzyme is synthesized in any cell normally making epinephrine. (*Pnmt* encodes phenylethanolamine N-methyltransferase, the enzyme that converts norepinephrine to epinephrine.) When crossed with appropriate tester strains, *Pnmt*-expressing cells and their descendants become beta-galactosidase–positive and thus can be readily identified and isolated. Early analyses indicate that epinephrine is synthesized by cells that give rise specifically to cardiac conduction cells, albeit a subset of these cells. Ongoing experiments will characterize the cell types. Parenthetically, our studies have generated a mouse that produces no epinephrine but shows normal levels of norepinephrine, thus allowing the specific roles of the two hormones to be dissected.

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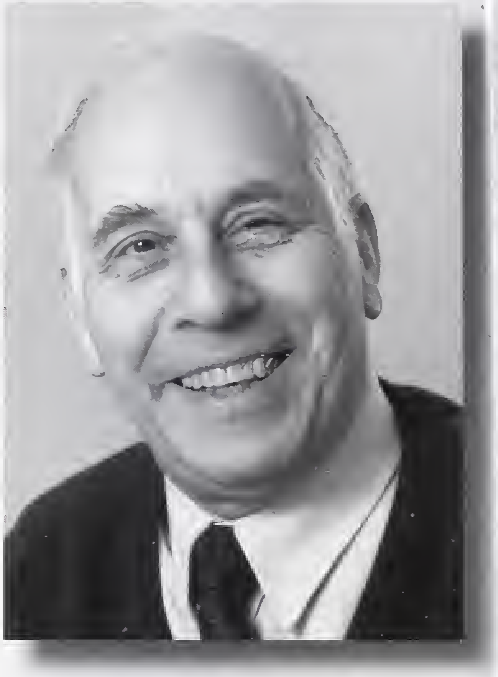
MAMMALIAN DEVELOPMENTAL GENETICS



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We carry out a functional analysis of genes that control critical steps of postimplantation development in the mammalian embryo. Transcription factors are major players in the cascade of molecular events that implement the body plan. We use a loss-of-function approach to study in detail the function of the LIM/homeodomain (*Lhx*) class of transcription factors and of factors that mediate their action. In addition, the group identifies novel genes involved in specific aspects of embryonic development and collaborates with other research teams on projects focusing on gene-altered mice.

Transcription factors that regulate mammalian development and factors that mediate their action

Bronstein, Chen, Crumity, Geum, Gorivodsky, Grinberg, Huang, E. Lee, W.K. Lee, Ma, Mailloux, Malik, Morales, Mukhopadhyay, Schindler, Teufel, Wang, Westphal, Williams-Simons, Wu, Zhao; in collaboration with members of the Agulnick, Bayarsaihan, Dawid, Downs, Hukriede, Kennison, Niehrs, Ogryzko, Oliver, Podtelejnikov, Rubenstein, Segal, and Yamashita laboratories

Over the years, our studies have shown that members of the *Lhx* gene family, encoding LIM-homeodomain–containing transcription factors, are involved in early patterning events in the development of the nervous system and in orchestrating organ assembly. More recently, we identified two gene families, termed *Ldb* and *Ssdp*, that encode obligatory cofactors of *Lhx* gene action. Our current work focuses on functions of the LIM-homeodomain proteins Lhx8 and Lhx2 and the LIM-domain–binding protein Ldb1 in embryonic development. Previously, we generated a strain of knock-out mice with a null-deletion of the LIM-homeobox gene *Lhx8*. Our initial analysis of the mutant mice revealed an important role for *Lhx8* in development of the palate. Given that the same gene is also abundantly expressed in the developing ventral forebrain, we recently examined the brain of our *Lhx8* null mice. The expertise of the Rubenstein laboratory greatly aided our work.

Development of cholinergic neurons in the ventral telencephalon is severely impaired in the knock-out mutants. As a result, the mice lack the nucleus basalis, a major source of cholinergic neuron input to the cerebral cortex. In addition, the number of cholinergic neurons in several other regions of the subcortical forebrain, including the caudate putamen, septum, and magnocellular preoptic nucleus, is reduced. Our marker analysis revealed that progenitor cells form in the absence of *Lhx8* gene function. However, the gene is essential for terminal differentiation of the majority of cholinergic neurons in the ventral telencephalon. The loss of telencephalic cholinergic projection neurons is a hallmark of neurodegenerative disorders such as Alzheimer's disease and is likely to play a role in the cognitive impairments of these patients. For this reason, the *Lhx8* null mutant mouse constitutes a valuable model for functional studies of cholinergic projection neurons in the context of memory and cognition.

Another ongoing study concerns the function of the LIM-homeobox gene *Lhx2* in brain development. Our initial *Lhx2* knock-out study had shown that the gene is essential for eye development and the formation of the telencephalon. More recently, we observed that development of the ventral diencephalon and pituitary is also impaired in the null mutant embryo, indicating an important additional role of *Lhx2* in forebrain development.

We have also continued our functional evaluation of protein complexes formed by LIM-homeodomain proteins and their associated cofactors. The Ssdp cofactors that we discovered are thought to form complexes with many transcription factors that are active during early stages of embryonic development. Whereas the vertebrate *Ssdp* gene family has several closely related members, the *Drosophila ssdp* gene is unique. Moreover, the existence of several mutant alleles of the gene makes the *Drosophila* system attractive for a genome-wide search for downstream genes whose transcription is regulated by Ssdp and associated proteins. Given that loss-of-function mutations in the *Drosophila ssdp* gene cause lethality at early pupal stages, we chose to focus our search on the late third instar larval stage, which precedes the pupal stage. We carried out the analysis in collaboration with the Oliver laboratory by using *Drosophila* genomic amplicon microarrays; we are currently evaluating the resulting wealth of data in an effort to identify direct targets of ssdp-mediated transcriptional regulation.

Earlier work, carried out in collaboration with the Niehrs laboratory, had shown that Dkk1, an inhibitor of canonical Wnt signaling, is an important mediator of transcriptional activity during vertebrate head induction. We have extended these observations by showing that head induction is based on a functional cooperation between Dkk1 and the BMP antagonist noggin.

In an unrelated project, we have identified FoxP4, a forkhead transcription factor involved in embryonic development, cell cycle regulation, and oncogenesis.

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Collaborative projects

Grinberg, Hasuike, Huang, E.J. Lee, Miyamoto, Westphal; in collaboration with members of the Chou, Enikolopov, Gold, Lamb, and Sibley laboratories*

In collaboration with the Lamb laboratory, we have identified in azoospermic patients a single nucleotide deletion in the *SYCP3* gene that causes a truncation of a protein required for chromosomal pairing during meiosis. The study represents an important advance in understanding the molecular basis of early meiotic arrest as a cause of nonobstructive azoospermia. Our experiments suggest that the truncated protein dominantly interferes with the function of the wild-type allele and thus causes male infertility.

Our group has also been involved in a diverse array of other collaborative projects aimed at revealing gene functions via knockout. The studies have shown that (1) the D5 dopamine receptor is involved in blood pressure regulation; (2) the vasopressin receptor 1a is a negative regulator of B cell receptor signaling; (3) nitric oxide acts as a negative regulator of cell proliferation in the adult mammalian brain; and (4) the glycose-6-phosphate transporter, deficient in glycogen storage disease type Ib, is an important immuno-modulatory protein.

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NUTRIENT CONTROL OF BACTERIAL GENE EXPRESSION

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Our goal is to understand how global patterns of bacterial gene expression are coordinated with nutrient availability. We continue to focus on the roles of (p)ppGpp, two regulatory nucleotide analogs related to GTP and GDP, but with a pyrophosphate esterified to the ribose 3′-hydroxyl. Nutrient limitation elevates (p)ppGpp, and nutrient sufficiency restores low basal levels, a mechanism that operates during starvation for amino acids, phosphate, nitrogen, or energy sources. Regulatory roles are assigned to (p)ppGpp; the elimination of (p)ppGpp can abolish regulation during starvation while the artificial elevation of (p)ppGpp (without starvation) mimics many regulatory effects of starvation. Responses to (p)ppGpp are a critical element in adaptive responses to starvation. We wish to understand the details of the molecular mechanisms whereby nutrient limitation governs (p)ppGpp metabolism and how regulation by (p)ppGpp works. Knowledge of these mechanisms is important for a basic understanding of the cell's interaction with nutrient availability. It is also of practical importance because an abundant literature links (p)ppGpp with bacterial pathogenicity in Gram-negative bacteria, synthesis of toxins and antibiotics in Gram-positives, and persistence of chronic infections in *Mycobacteria tuberculosis*, which occurs in the majority of the world's population.

Discovery and structural documentation of an intrinsic protein switch for reciprocal control of (p)ppGpp synthesis and degradation

Mechold, Murphy, Cashel; in collaboration with Hilgenfeld, Hogg*

The balance of rates of (p)ppGpp synthesis and degradation determines (p)ppGpp's level as a nutritional stress signal. Regulation is achieved without simultaneously increasing both synthesis and degradation, which would be a futile waste of ATP. Activation of (p)ppGpp synthesis can be triggered when the supply of any aminoacyl tRNA fails to keep up with the demands of protein synthesis. An example is amino acid starvation leading to a decline in aminoacyl-tRNA and an increase in uncharged tRNA. Excess uncharged tRNA is then able to bind preferentially to ribosomal acceptor (A) sites dictated by now available "hungry" mRNA codons. We wish to understand the molecular details of the interaction between RelA, the ribosome, and the requirement for mRNA-specified uncharged tRNA binding.

Degradation of (p)ppGpp is also controlled by sensing starvation for nutrients other than amino acids. The degrading enzyme is a (p)ppGpp-specific 3′-pyrophosphoryl hydrolase, called SpoT in *E. coli* and most other enteric bacteria. Historically, we now appreciate that, instead of separate but homologous enteric proteins for synthesis (RelA) and degradation (SpoT), only a single Rel/Spo homolog (Rsh*)* protein harboring both functions is present in most bacteria.

Last year, we reported finding that the Rsh protein from *Streptococcus* with its two catalytic domains in the N-terminal half of the protein (NTD) could exist in two activity states, depending on the presence of the C-terminal half-protein (CTD). In state 1 hydrolase is off, whereas synthetase is on; in state 2 hydrolase is on while synthetase is off. Hydrolase activity can change 50-fold, whereas synthetase activity varies about 15-fold. These effects are quantitatively consistent with findings made by other researchers using the full-length Rsh protein from *M. tuberculosis* on ribosomes bound with codon-specified uncharged tRNA. Our mutant mapping of hydrolase and synthetase catalytic domains found them to be nonoverlapping, verifiable with activity assays of peptides. We found unusual mis-sense mutants of one activity to be reactivated by reassembly with the missing domain, even though the CTD was absent. Subsequent isolation of domain-specific suppressors hinted that regions in one domain could regulate the activity of the other domain, independent of the CTD. We conclude that the switch leading to reciprocal regulation of opposing catalytic activities is an intrinsic "hard wired" feature of the NTD that could be changed by special NTD mutants, by NTD-CTD interactions, or by signals operating on the CTD.

This year, an eight-year collaboration to determine the NTD structure has reached fruition by solving crystal structure at a resolution of 2.1 Å. The hydrolase structure closely resembles cyclic phosphodiesterases with a characteristic metal-dependent phosphohydrolase fold consisting of an up-down-up-down bundle of four alpha helices together with loop and a three-helix bundle. The synthetase structure, with its catalytic site 30 Å away from the hydrolase site, is a five-stranded mixed beta sheet sandwiched by five alpha helices. With the placement of two of the helices, the structure is similar to the nucleotidyl transferases superfamily, with the human DNA polymerase beta, polyA polymerases, and terminal deoxynucleotidyl transferases representing the closest structural examples. The structural relatedness is not evident from the linear amino acid sequence. The structure subjected to analysis consists of a dimer, with each monomer in a different conformation. Each monomeric conformation can be deduced to represent each of the two reciprocal activity states. We infer that allosteric transitions between the two monomeric conformations are attributable to ligand binding. Many unanswered questions remain regarding the predicted roles of individual residues. We also do not know how the regulatory CTD, uncharged tRNA, ribosomal binding, and environmental signals other than amino acid starvation provoke the transition between the two activity states.

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Regulation of (p)ppGpp mediated by the SpoT protein

Gal, Murphy, Cashel; in collaboration with Schneider

We are also searching for mechanisms that lead to (p)ppGpp regulation through involvement of the SpoT protein, particularly with the CTD region that we know, from deletion studies, has regulatory functions. One approach involves characterization of spontaneous CTD region mutants found repeatedly among identical populations of *E. coli* B strains grown for over 20,000 generations by repeated overnight cultures in glucose minimal medium under standardized conditions. Researchers in Grenoble and at the NIH are characterizing these mutants. Typically, the new *spoT* CTD alleles appear to provide a 14 percent growth advantage over the wild type after a single overnight culture when introduced into the ancestral (generation 0) strain. In these evolving cultures, the *spoT* alleles appear to arise as a consequence of earlier spontaneous *topA* mutations, which also confer a growth advantage. It is unclear whether the growth advantage occurs at the level of steady-state growth or stationary-phase transitions. The onset of the stringent response to amino acid starvation appears to be accelerated in such mutants, but details remain to be characterized.

Using a commercial (Bacteriomatch) bacterial two-hybrid system with the SpoT CTD as bait for screening an *E. coli* random fragment library, we have also searched for proteins interacting with

SpoT protein. Our work led to the finding that protein fragments encoded by *yrdA* and *fepE* genes can bind to the SpoT CTD, verifiable by co-immunoprecipitation with a full-length SpoT-MalB fusion protein. The function of the *yrdA* gene is unknown, but its location is interesting. Its promoter is embedded in the upstream activation element of a very strong (*rrnD*) ribosomal RNA promoter region (*rrnD*) with transcripts differing from those of *r*RNA. This raises the possibility that abundance of *yrdA* transcripts might be reciprocally regulated by *rrnD* transcription, which is, in turn, known to be negatively regulated by ppGpp. We found that *yrdA::lacZ* fusion activities increase about 50-fold when *rrnD* transcripts are limited as a consequence of elevation of ppGpp. This finding led us to hypothesize that YrdA protein might act in a feedback regulatory circuit, with SpoT as a sensor of rRNA transcription. Induction of ppGpp is known to follow a damped harmonic pattern suggestive of such a circuit. Thus, high levels of ppGpp indirectly lead to overexpression of YrdA, which could promote its binding to SpoT CTD and then provoke lower ppGpp levels by either stimulating SpoT hydrolase or inhibiting SpoT synthetase. However, artificially overexpressing YrdA has few effects, including only a modest slowing of the turnoff of a *rrnB-lacZ* fusion during entry into stationary phase. This behavior weakly supports the hypothetical existence of the feedback loop. Deleting *yrdA* has no obvious phenotype even in indirect assays of (p)ppGpp levels.

The role of FepE binding to SpoT is also uncertain. The *E. coli* K-12 *fepE* gene encodes a membrane protein homologous to an *S. typhimurium* protein that functions as a determinant of lipopolysaccharide O-antigen chain lengths. Long chain lengths are associated with increased virulence. Our strain of *E. coli* is defective in O-antigen synthesis because of an insertion element inactivation of the synthetic pathway; we are thus unable to verify this function of FepE. We have not found a dramatic phenotype accompanying either overexpression or deletion of the hydrophilic loop of FepE. Nevertheless, a function of FepE in *E. coli* analogous to *S. typhimurium* might explain of why deleting the *spoT* gene along with *relA* diminishes *E. coli* pathogenicity.

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PATTERNING NEUROGENESIS IN THE ZEBRAFISH EMBRYO

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The formation of a healthy nervous system involves the regulated production of neurons during the course of embryonic development. By examining genetically how a relatively simple pattern of early neurons is established in the zebrafish neural plate, we have identified mechanisms that determine where and when cells are permitted to become neurons. In the past year, our studies have shown that HER5 (Hairy Enhancer of split Related gene-5) plays an essential role in preventing neurogenesis at the midbrain-hindbrain boundary where non-noneuronal cells can then maintain the function of the isthmic organizer. Screens for mutants with an aberrant pattern of early neurons led to the identification of *headless* (*hdl*) mutants that have a rostral expansion of trigeminal ganglia and of *mind bomb* (*mib*) mutants that have an unusually high density of neurons in otherwise normal locations. Analysis of the *hdl* mutant has provided novel insights into how the neural tube is divided into discrete compartments along the rostro-caudal axis while analysis of *mib* has led to the identification of a novel component of the Notch signaling pathway.

Inhibition of TCF3-mediated repression by Wnts promotes caudal fates in the neurectoderm

Itoh, Chitnis; in collaboration with Dorsky, Moon

In a screen for zebrafish mutants with an abnormal pattern of early neurons, the *headless* (*hdl*) mutant was identified by its aberrant rostral expansion of trigeminal neurons. Positional cloning of *hdl* revealed that the mutant phenotype is the result of a loss-of-function mutation in a zebrafish homolog of T-Cell Factor 3 (TCF3), a member of the tcf/lef transcription factor family that had previously been suggested as playing an essential role in regulating expression of genes activated by Wnt signaling. Analysis of the *hdl* mutant showed that the rostral expansion of trigeminal neurons is just one consequence of a more general posteriorization defect, for which loss of relatively rostral neural tissues, like the forebrain and eyes, is accompanied by expansion of relatively caudal domains, like the midbrain-hindbrain boundary region and adjacent trigeminal neurons. Furthermore, analysis showed that the mutant phenotype results from the loss of the essential function of Hdl as a transcriptional repressor (Kim *et al.*, 2000).

Previous studies had shown that exaggerated Wnt signaling at the shield stage in zebrafish can phenocopy the maternal zygotic (MZ) *hdl* phenotype. Furthermore, it had been suggested that a rostro-caudal gradient of Wnt signaling contributes to specification of discrete compartments of the neuroectoderm along the rostro-caudal axis. Consistent with these studies, our analysis suggested that *hdl* encodes a Tcf3 homolog that plays an essential role in repressing the expression of transcription factors that define relatively caudal domains in the neurectoderm in response to posteriorizing factors like Wnts. We interpreted the phenotype of MZ *hdl* mutants to suggest that, in the absence of repression provided by Hdl, posteriorizing factors aberrantly drive expression of transcription factors that define caudal domains in relatively rostral compartments, which causes transformation to more caudal fates.

Consistent with this interpretation, a knock-down of Wnt8 function by morpholinos led to a phenotype that was exactly the opposite of *hdl,* in which a reduction of caudal domains was accompanied by an expansion of relatively rostral domains. This suggested that Wnt8 is an essential posteriorizing factor. Given our conclusion that *hdl* was responsible for repressing the response to Wnt signals that drive expression of caudal genes, we predicted that a knock-down of Wnt8 would diminish the expansion of caudal domains observed when *hdl* function is reduced. However, when Wnt8 function was knocked-down in an *hdl* mutant background, we observed no obvious reduction in the rostral expansion of caudal domains, suggesting that Wnt8 is not essential for directly driving expression of genes that define caudal domains. Instead, it may indirectly facilitate expression of caudal genes by inhibiting the function of Hdl, a repressor that normally prevents expression of relatively caudal genes. To test such a possibility, we examined how Wnts affect *hdl* function. We showed that the eyeless phenotype in MZ *hdl* mutants can be rescued by injecting mutant embryos with the appropriate amount of *hdl* mRNA at the one-cell stage. However, the co-injection of embryos with *Wnt1* mRNA prevents the rescue, showing that Wnt signaling may indeed be able to inhibit *hdl* function. In the canonical Wnt signaling pathway, Wnts mediate their effects through beta-catenin. Given that the Hdl transcription factor has a beta-catenin binding domain at its N-terminal, we hypothesized that it is the Wnt signaling domain that influences Hdl function. Consistent with our hypothesis, when Wnt1 RNA was co-injected with RNA encoding a truncated form of Hdl lacking its N-terminal beta-catenin binding domain, it was unable to prevent the rescue of MZ hdl mutant embryos (Dorsky *et al*., 2003). This observation supports the conclusion that Wnts can inhibit function of Hdl as a repressor and that the inhibition requires the Hdl N-terminal beta-catenin binding domain. Furthermore, our observations suggest that, in the developing embryo, a gradient of Wnt activity may differentially inhibit repression mediated by TCF3 homologs such as *hdl* and establish a pattern of graded repression that acts in concert with an opposing gradient of posteriorizing activity mediated by factors like FGF. Together, the combination of positive and negative regulation may help establish discrete compartments of gene expression along the rostral-caudal axis of the neural plate.

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The bHLH transcription factor Her5 links patterning to regional inhibition of neurogenesis at the midbrain-hindbrain boundary

Itoh, Chitnis; in collaboration with Bally-Cuif

The midbrain–hindbrain boundary (MHB) domain of the vertebrate embryonic neural plate is characterized by the absence of early neurons. We showed in zebrafish embryos that it is difficult to induce neurogenesis in this domain by the ectopic expression of the proneural gene neurogenin1 (ngn1). When neurogenesis is forced by ectopic expression of relatively high levels of ngn1, it interferes with the continued expression of genes defining MHB identity. Expression of the bHLH Hairy/E(spl)–related factor Her5 prefigures and then precisely delineates the neuron-free zone at the MHB in which neurogenesis is suppressed throughout embryonic development. Using morpholino knock-down and conditional gain-of-function assays, we demonstrated that Her5 is essential to prevent neuronal differentiation and to promote cell proliferation in a medial compartment of the of the MHB neuron-free zone. Together, our results suggest the existence of a mechanism that actively inhibits neurogenesis at the MHB, a process that might ultimately be necessary to maintain integrity of the MHB domain. Furthermore, our study shows that Her5 is a partially redundant component of the inhibitory process that helps translate early axial patterning information into a distinct spatiotemporal pattern of neurogenesis and cell proliferation within the midbrain–hindbrain domain.

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The ubiquitin ligase Mib is essential for efficient activation of Notch signaling by Delta

Itoh, Palardy, Maust, Yeo, Chitnis; in collaboration with Ariza-McNaughton, Chandrasekharappa, Jiang, Kim, Lewis, Lorick, Oda, Weissman, Wright

Zebrafish *mind bomb* (*mib*) mutants display a severe neurogenic phenotype characterized by an excess of early neurons and a reduction of neurons that are generated relatively late in development. Though previous studies have suggested that *mib* is likely to encode an essential component of the Notch pathway, the molecular nature of mib had remained elusive, and it was not known how it contributes to Notch signaling.

Notch is a one-pass transmembrane receptor that is synthesized as a single peptide. Furin-mediated cleavage of the peptide creates two fragments that are held together in the mature receptor as a heterodimer. The extracellular fragment mediates interactions with the ligand Delta. Binding to Delta makes the Notch receptor vulnerable to metalloproteases that cleave Notch at a site outside its transmembrane domain, facilitating the removal of the Notch extracellular domain (NECD). The membrane-bound Notch fragment that remains on the adjacent cell following removal of the NECD is a constitutive substrate for gamma-secretases that are responsible for an intramembranous cleavage. The cleavage releases the Notch intracellular domain, which is the active fragment of the Notch receptor. Though many aspects of Notch signaling have been studied for a number of years, the steps by which the Notch ligands activate the Notch receptor remain poorly defined.

Positional cloning of *mib* revealed a novel gene in the Notch pathway that encodes a RING E3 ubiquitin ligase, an enzyme that plays a key role in ubiquitylation of specific substrates. Analysis of its role in Notch signaling has revealed that Mib interacts with the intracellular domain of Delta to promote its ubiquitylation and internalization. Paradoxically, Mib-mediated internalization of the Notch ligand appears to be essential in the Delta-expressing cell for efficient activation of Notch in neighboring cells. The mechanism by which Delta internalization facilitates Notch activation in neighboring cells appears unclear at this time.

We are now investigating whether Mib-mediated ubiquitylation and internalization of Delta are required primarily to facilitate access of metalloproteases to the Notch extracellular domain and/or to facilitate removal of the NECD following cleavage by proteases. Previous studies have shown that clustering of Delta dramatically increases its ability to interact with and activate Notch. In this context, we are also investigating whether Mib contributes to Notch signaling by promoting the clustering of Delta.

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PHYSIOLOGICAL, BIOCHEMICAL, AND MOLECULAR GENETIC EVENTS OF RECOGNITION AND RESOLUTION OF RNA-DNA HYBRIDS

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Our research is directed at understanding the processes involved in cellular DNA replication and the relationship of HIV replication to DNA replication and at using the resultant information for therapeutic purposes. We are examining the formation and resolution of RNA-DNA hybrids that emerge during DNA replication or transcription. Ribonucleases H participate in the removal of the RNA of the RNA-DNA hybrids and are intimately related to DNA replication in cells; in HIV replication, they play a role during the conversion of the RNA genome of the virus to DNA. Using similar protein architecture, RNases H of cells and HIV share common enzymatic mechanisms of RNA cleavage. Drugs to alter levels of specific disease-related genes are under development to take advantage of RNases H within the cell. Regulated expression of RNases H could enhance the efficacy of the drugs. We use molecular genetic, biochemical, and mouse animal models in our research efforts.

RNase H1 requirement for embryogenesis in mice

Cerritelli, Crouch; in collaboration with Love

Mouse and human RNases H1 have an N-terminal mitochondrial localization sequence. Using transient transfection assays, we demonstrated mitochondrial targeting by the 26–amino acid leader of the mouse RNase H1. We created an *Rnaseh1* knockout to test for effects on mitochondrial function *in vivo*. We found that *Rnaseh1*$^{-/-}$ mice arrest growth *in utero* at day E8.5 and exhibit defective respiratory function and massive apoptotic cell death, which are both direct results of a failure in mitochondrial DNA replication. Two mechanisms have been described for replication of mitochondrial DNA, one of which suggests a prominent role for RNase H1 during replication while the second has no apparent use for RNase H1. Although we favor a single mechanism dependent on RNase H1, two modes of mitochondrial DNA replication may exist, and sequential activation of each mode during development would indicate that RNase H1 is required for the earlier system to be established.

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Mechanism of action of mouse and human RNases H1

Gorshkova, Gaidamkov, Cerritelli, Crouch; in collaboration with Cazenave

A few years ago, we discovered that eukaryotic RNases H1 have an RNase H domain similar in size, amino acid sequence, and structure to that we reported for *E. coli* RNase HI. Eukaryotic RNases H1 have an additional domain connected to the N-terminus of the RNase H domain that can bind duplex RNAs even in the absences of the RNase H domain. We have now shown that this "extra" dsRNA-binding domain aids in dimerization of the full-length protein when an RNA-DNA substrate is present. Aptamers are nucleic acids that are selected for performing various activities normally attributed to enzymes. Usually, RNA aptamers are generated, but, given that human RNase H1 (and most other eukaryotic RNases H1) binds to duplex RNAs, we have generated DNA aptamers that bind to and inhibit human RNase H1. Interestingly, several homologs of the human protein are also inhibited. This new tool will be useful for dissecting the mechanism by which the RNases H1 interact with their substrates.

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Eukaryotic RNase H2 activation

Jeong, Crouch

Bacterial and archael RNases HII are active as a single polypeptide chain, and several critical residues have been shown to be a part of the active site. The eukaryotic RNase H2 enzyme is considerably larger than RNases HII, yet the orthologous eukaryotic polypeptide is similar in size and sequence to the smaller RNases HII. We have expressed the RNase H2 polypeptide encoded by human, mouse, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* in *E. coli* and uniformly found the protein to have no RNase H enzymatic activity. It seems that eukaryotic RNases H2 require some modification and/or that more than one subunit is necessary for RNase H2 activity. From *S. cerevisiae* cells, we have affinity-purified the RNase H2 polypeptide of *S. cerevisiae* and find two proteins copurifying in a complex. We determined the identities of the proteins with mass spectrometry. RNase H2 activity in *S. cerevisiae* strains deleted for either of the two genes is absent, suggesting that both of the proteins may be components of the active enzyme. Coexpression in *E. coli* of the three proteins identified as components of RNase H2 generates an active RNase H2. Thus, the three proteins are necessary and sufficient for forming the three-subunit RNase H2.

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Biomolecular interactions

Gorshkova, Couch; in collaboration with Backer, Bonifacino

Using surface plasmon resonance, we are able to measure in real-time biomolecular interactions and compare their relative efficiencies. In collaboration with SibTech, Inc., we have studied another type of interaction that could prove useful as a means of specifically targeting drugs: the use of naturally occurring interactions between proteins or portions of proteins to form stable complexes. Fused to one of the two partners is a protein with a receptor on only a limited type of cell and that will therefore deliver the drug to those cells with the receptor. For the system to work, the noncovalent interaction bringing the two components together must be stable. Surface plasmon resonance studies indicate a highly stable complex between the S-peptide and S-protein of human RNase A even when the ligand vascular endothelial growth factor (VEGF) was attached to the S-peptide. Indeed, delivery to cells expressing the VEGF receptor was found to be remarkably efficient.

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MOLECULAR GENETICS OF EMBRYOGENESIS IN *XENOPUS* AND ZEBRAFISH

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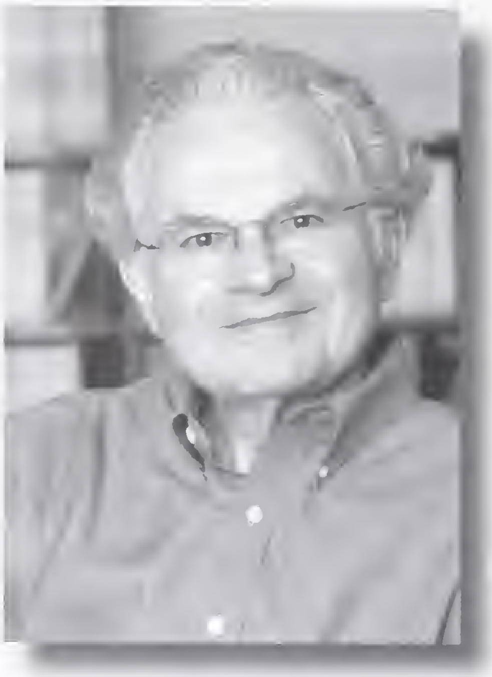

The laboratory is engaged in studies of molecular-genetic mechanisms of early vertebrate development, using the frog *Xenopus laevis* and the zebrafish *Danio rerio* as experimental systems.

The small GTPase Rac mediates Wnt signaling in controlling gastrulation movements

Habas, Dawid; in collaboration with He

Wnt signaling is known to control gastrulation movements in different vertebrate embryos. Gastrulation movements involve a branch of the Wnt pathway, a branch that is mediated by frizzled receptors and the Dishevelled (Dsh) protein but does not result in activation of beta-catenin. Recently completed experiments show that activation of the small GTPase Rac is also required for gastrulation movements; we previously showed the same requirement for the related factor Rho. Rac activation is mediated by Wnt signaling through the function of Dsh, but the structural requirements in Dsh for Rho and Rac activation differ. Inhibition of either Rho or Rac activation alone leads to failure of gastrulation, indicating that the independent activation of both GTPases downstream of Wnt signaling is required for early development.

Habas R, Dawid IB, He X. Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes Dev* 2003;17:295-309.

The control and function of *Lim1* in gastrulation

Hukriede, Habas, Rebbert, Takahashi, Dawid; in collaboration with Andreazzoli, Tam, Weeks, Whitman

The *Lim1* gene encodes a LIM-homeodomain protein that is expressed in the organizer region of all vertebrate embryos. Experiments using transient expression of *Lim1* constructs showed that activin/nodal regulation of *Lim1* expression is mediated by Sma and FAST sites located in the first intron. These results clarify the regulatory machinery that ensures activation of *Lim1* at the late blastula stage.

We studied the function of *Lim1* in *Xenopus* by using antisense oligonucleotide depletion techniques. *Lim1*-depleted frog embryos failed to form head structures, providing a phenocopy of *Lim1*-deficient mice that had been studied previously by others. Most organizer-specific gene expression was retained in the affected embryos, but cell movements characteristic for gastrulation were impeded.

The defect was traced to impaired expression of paraaxial protocadherin (PAPC), an adhesion molecule known to be involved in gastrulation. Complementary studies in the mouse showed that *Lim1*$^{-/-}$ cells fail to participate in gastrulation movements when transplanted into wild-type embryos. Together, these studies indicate that control of gastrulation movements is a major function of the *Lim1* gene in different vertebrate embryos.

Hukriede NA, Tsang TE, Habas R, Khoo PL, Steiner K, Weeks DL, Tam PP, and Dawid IB. Conserved requirement of Lim1 function for cell movements during gastrulation. *Dev Cell* 2003;4:83-94.

Watanabe M, Rebbert ML, Andreazzoli A, Takahashi N, Toyama R, Zimmerman S, Whitman M, Dawid IB. Regulation of the Lim-1 gene is mediated through conserved FAST-1/FoxH1 sites in the first intron. *Dev Dyn* 2002;225:448-456.

Sef, a novel feedback inhibitor of FGF signaling

Tsang, Kudoh, Dawid; in collaboration with Friesel

Fibroblast growth factors constitute a family of signaling factors that regulate many different developmental and physiological processes, and mutations in FGF receptors are associated with several conditions in humans. While the FGF signal transduction pathway has undergone extensive study in many systems, we were able to isolate a novel feedback inhibitor of the pathway by observing a gene with Similar Expression to FGFs (Sef) in zebrafish. The group of C. and B. Thisse independently identified Sef, which is a transmembrane protein whose expression is controlled by FGF signaling. Its role, as studied by gain-of-function and loss-of-function approaches, is to attenuate the FGF signal in the embryo. Thus, Sef joins the ranks of many factors identified in various pathways that have a role in limiting the extent or range of their cognate signaling pathway within the organism.

Tsang M, Friesel R, Kudoh T, Dawid IB. Identification of Sef, a novel modulator of FGF signaling. *Nat Cell Biol* 2002;4:165-169.

Interplay of different signaling pathways in patterning the neural ectoderm in zebrafish

Kudoh, Dawid; in collaboration with Wilson

The neural ectoderm is patterned during gastrulation by interaction between several signaling pathways. We have studied the regulatory interrelationships between these signaling factors in the zebrafish. We first studied the interaction between the retinoic acid (RA), FGF, and Wnt pathways in specifying trunk neural ectoderm in the zebrafish and showed that RA is the downstream effector of these pathways. More recently, we studied the initial specification of different anteroposterior states in the neural ectoderm and found that combinatorial action of the FGF and BMP pathways define these states during gastrulation. BMP does not simply antagonize neural development, as generally assumed, but does so only in the absence of FGF. In FGF's presence, BMP acts as a caudalizing factor so that regions high in both BMP and FGF signaling give rise to tail structures. In the absence of FGF, BMP signaling specifies epidermis. These studies expand our knowledge of the anteroposterior specification in the prospective neural ectoderm of the zebrafish embryo.

Kudoh T, Wilson SW, Dawid IB. Distinct roles for FGF, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* 2002;129:4335-4346.

Foxi1 is required for ear development in the zebrafish

Kudoh,[b] Dawid; in collaboration with Fritz

The development of the ear in the zebrafish requires the action of several signaling pathways and of a series of transcription factors that regulate gene expression in the prospective otic vesicle. In matching a cDNA clone obtained in an expression screen in our laboratory with a mutant zebrafish line affected in ear development that was discovered by Solomon and Fritz, we showed that Foxi1 is required for ear development. Foxi1 is a member of the forkhead class of transcription factors, a large family of important developmental regulators. Our studies help clarify the genetic cascade that promotes the early specification of the otic vesicle in the zebrafish, and there are strong reasons to believe that Foxi1 has a similar role in all vertebrates.

Solomon KS, Kudoh T, Dawid IB, Fritz A. Zebrafish foxi1 mediates otic placode formation and jaw development. *Development* 2003;130:929-940.

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CONTROL OF GENE EXPRESSION DURING DEVELOPMENT

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During development and differentiation, genes become competent to be expressed or are stably silenced in an epigenetically heritable manner. This selective activation/repression of genes leads to the differentiation of tissue types. Our group is interested in the molecular mechanisms that lead to the heritable transmission of the silenced state. To that end, we are studying the mechanism of gene silencing by the *Polycomb* group genes (PcG) in *Drosophila*. The PcG genes encode a diverse group of proteins known to be important for silencing homeotic genes during development in organisms as diverse as *Drosophila* and humans. Many PcG genes encode chromatin-associated proteins that are thought to silence transcription through modification of histones and formation of inactive chromatin. PcG proteins act through *cis*-acting DNA sequences called Polycomb group Response Elements (PRE). PREs are thought to recruit PcG protein complexes to the DNA. Given that there are different types of PcG protein complexes, there may be different types of PREs. A major focus of our laboratory is to characterize fully the sequences and DNA binding proteins required for the function of a well-defined PRE.

Polycomb group response elements require the activity of many DNA binding proteins

J.L. Brown, Grau, Kassis

We have completed a detailed study of one PRE from the segmentation gene *engrailed*. Within a 139-bp minimal PRE, we have identified eight protein-binding sites. Our functional data have shown that at least five of the sites are required for PRE activity. One of the proteins that binds to this PRE is the product of the *pleiohomeotic* (*pho*) gene, a known PcG gene. *pho* encodes a *Drosophila* homolog of the mammalian zinc-finger transcription factor Yin Yang 1 (YY1). Another binding site is the sequence GAGAG, which is known to bind to two *Drosophila* proteins, GAGA factor and pipsqueak. Pho has two binding sites and two GAGAG sequences within the *engrailed* PRE. The proteins that bind to the other four sites have yet to be identified. Isolation of these proteins is a major focus of our laboratory. We are using a yeast one-hybrid screen and biochemical methods to isolate the other DNA binding proteins involved in this important process. We have recently identified a family of zinc-finger proteins that bind to one of the sequences important for PRE activity and are currently investigating which of these family members may be involved in Polycomb group repression. Current experiments suggest that at least five of the proteins bind to the minimal PRE *in vitro*. By undertaking chromatin-immunoprecipitation experiments, we will generate antibodies to these proteins to determine which ones are bound to the PRE *in vivo*. Other experiments will attempt to address the role of the proteins by depleting the proteins that use RNAi and testing whether PRE function is compromised.

Americo J, Whiteley M, Brown JL, Fujioka M, Jaynes JB, Kassis JA. A complex array of DNA-binding proteins required for pairing-sensitive silencing by a polycomb-group response element from the Drosophila *engrailed* gene. *Genetics* 2002;160:1561-1571.

The role of pho-like in Polycomb group repression

JL Brown, Fritsch, Müller, Kassis

The identification of Pho binding sites in many different PREs suggests that Pho is a key component for the recruitment of PcG protein complexes to the DNA. If so, then the phenotype of *pho* mutants should be severe derepression of homeotic genes. Curiously, homeotic genes in *pho* mutants are only mildly derepressed. The sequencing of the *Drosophila* genome permits a possible explanation of this apparent paradox. Given that another YY1 homolog exists in *Drosophila*, we are studying the function of this gene, which we call *pho-like*, and have generated mutants. Flies mutant for *pho-like* are viable, but the females are sterile. Homozygous *pho-like* mothers lay eggs that are fertilized but fail to undergo nuclear divisions. Thus, Pho-like must be deposited in the egg by the mother for development to occur. Organisms that are double-mutant for *pho* and *pho-like* (from mothers and fathers heterozygous for these two mutations) develop into larvae and have much more severe derepression of homeotic genes than *pho* mutants alone, showing that *pho-like* enhances the *pho* phenotype. In fact, the derepression of homeotic genes seen in imaginal disks from *pho-like*, *pho* double mutants is comparable to that seen in many other PcG mutants, suggesting that Pho and Pho-like play redundant but highly important roles in PcG repression.

Pho-like binds to Pho binding sites *in vitro*, and *pho-like*, *pho* double mutants show more severe misexpression of homeotic genes than the single mutants. But how do the two proteins function? Given that Pho and Pho-like are DNA binding proteins, one hypothesis is that they may recruit or anchor PcG protein complexes to the DNA. To investigate this possible function, we examined the distribution of five PcG proteins on polytene chromosomes from *pho-like*, *pho* double mutants. The five PcG proteins are not DNA binding proteins but are associated with specific sites on wild-type polytene chromosomes. We reasoned that if Pho and Pho-like are required for anchoring PcG protein complexes to the DNA, then association of PcG chromatin proteins with chromosomal sites might be lost. Our results showed that the PcG proteins Pc, Psc, Scm, E(z), and Ph remain bound to polytene chromosomes at most sites in the absence of Pho and Pho-like. At a few chromosomal locations, however, some of the PcG proteins were no longer present in the absence of Pho and Pho-like. The data suggest the presence of both Pho-like/Pho-dependent and Pho-like/Pho-independent PREs. We posit that, at most genes, both types of PREs exist. Thus, in polytene chromosomes, only a subset of PcG protein complexes may be lost at each genomic location. Alternatively, Pho and Pho-like may act in concert with other PcG proteins but are not required for their association with the DNA.

Brown JL, Fritsch C, Müller J, Kassis JA. The drosophila *pho-like* gene encodes a YY1-related DNA binding protein that is redundant with *pleiohomeotic* in homeotic gene silencing. *Development* 2003;130: 285-294.

How do distant regulatory elements interact with a promoter?

Kelly, Gordon, A. Brown, Kassis

Regulatory sequences in eukaryotic genes can be located many tens of kilobases away from the promoter. How do these distant regulatory sequences activate or repress the promoter? One model holds that proteins bound to the distant enhancer interact with proteins bound near the promoter, causing the intervening DNA to form a loop. One of the fragments of DNA from the *Drosophila engrailed* gene that has PRE activity also can cause interactions between distant DNA fragments. We are currently trying to understand the function of this DNA *in vivo* by deleting it from the endogenous *engrailed* gene. Our results suggest that the fragment of DNA facilitates interactions between a distant enhancer involved in positively regulating engrailed expression in the wing and the engrailed enhancer. Thus, the fragment of DNA that, in some cases, acts as a negative regulatory element (a PRE) can, in fact, act as a positive regulatory element in other contexts. Our results are consistent with the notion that the role of this element is to facilitate interactions between distant enhancers or silencers with promoter elements.

Kassis JA. Pairing-sensitive silencing, polycomb group response elements, and transposon homing in *Drosophila. Adv Genet* 2002;46:421-438.

Transposon homing

Kelly, Gordon, Kassis

Instead of inserting randomly in the genome like other P-based transposons, P-element–based transposons that contain regulatory DNA from the *Drosophila engrailed* gene (*P[en]*) insert near the endogenous *engrailed* gene about 5% of the time. The phenomenon has been called "transposon homing" because the DNA present in the transposon directs it to insert ("home") near its parent locus. Only two other cases of transposon homing have been described. DNA from the bithorax complex and the *linotte* gene also cause transposon homing to their parent genes in *Drosophila.* The mechanism of homing is unknown, but it presumably involves the action of proteins bound to the transposon, causing the localization of the transposon to its parent genomic region. It is not known whether similar proteins are responsible for the homing at the three loci. It has been proposed that PREs might be one type of regulatory element that functions in transposon homing. We have tested this proposal by deleting a PRE present in *P[en]* and discovered that deletion of the PRE did not cause a loss of *P[en]* homing. The implication is that PcG proteins may not be involved in transposon homing at *engrailed.*

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REGULATION OF HOMEOTIC GENES IN *DROSOPHILA*

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Der-Hwa Huang, PhD, *Guest Researcher*

Our goal is to understand the regulation of homeotic gene function in *Drosophila*. The homeotic genes specify segmental identities in *Drosophila* at both the embryonic and adult stages. They encode homeodomain-containing transcription factors that control cell fates by regulating the transcription of downstream target genes. The homeotic genes are expressed in precise spatial patterns that are crucial for the proper determination of segmental identities. Both loss of expression and ectopic expression in the wrong tissues lead to changes in segmental identities. These changes in identity provide a powerful assay to identify the *trans*-acting factors that regulate the homeotic genes and the *cis*-acting sequences through which they act. Both the homeotic genes and the *trans*-acting factors that regulate them are conserved between *Drosophila* and man. In addition to many conserved developmental genes, at least half the disease and cancer-causing genes in man are conserved in *Drosophila*, making *Drosophila* a particularly important model system for the study of human development and disease.

Cis-acting sequences required for transcriptional regulation of the *Sex combs reduced* homeotic gene

Kennison, Cooper

Assays in transgenes in *Drosophila* have previously identified *cis*-acting transcriptional regulatory elements from the homeotic genes. The assays have identified both tissue-specific enhancer elements as well as *cis*-regulatory elements that are required for the maintenance of activation or repression throughout development. While these transgene assays have been important in defining the structure of the *cis*-regulatory elements and in identifying *trans*-acting factors that bind to them, their functions within the context of endogenous genes are still not well understood. We have used a large number of existing chromosomal rearrangements in the *Sex combs reduced* homeotic gene to investigate the functions of the *cis*-acting elements within the endogenous gene. These chromosomal rearrangements identified an imaginal leg enhancer about 35 kb upstream of the *Sex combs reduced* promoter. This imaginal leg enhancer can activate transcription not only of the *Sex combs reduced* promoter that is 35 kb distant but also of the *Sex combs reduced* promoter on the homologous chromosome. This *trans*-activation was first observed for the homeotic gene *Ultrabithorax* and named transvection. Characterization of the chromosomal rearrangements also revealed that two genetic elements about 70 kb apart in the *Sex combs reduced* gene must be in *cis* to maintain proper repression. When not physically linked to each other, these elements interact with elements on the homologous chromosome and cause derepression of its wild-type *Sex combs reduced* gene. To validate our model, we have characterized a transposable element insertional mutation that was isolated 50 years ago and has highly unusual genetic properties. The transposable element is inserted about 150 kb upstream of the *Sex combs reduced* promoter, and we believe that the unusual genetic properties of the insertion derive from its ability to mimic the endogenous genetic elements required for transcriptional repression. We have identified the transposable element as the *Drosophila Springer* retrotransposon. We have used an unlinked genetic suppressor of *Springer* to show that the unusual genetic properties are actually attributable to the *Springer* insertion. We have identified a 400 base pair region of the *Springer* retrotransposon that functions as a homeotic repression element in a transgene assay. We believe that comparisons between the *Springer* sequences and the sequences of the endogenous elements should reveal target sites that interact with the *trans*-acting factors.

Kennison JA, Southworth JW. Transvection in Drosophila. *Adv Genet* 2002;46:399-420.

Southworth JW, Kennison JA. Transvection and silencing of the *Sex combs reduced* homeotic gene of *Drosophila melanogaster*. *Genetics* 2002;161:733-746.

Trans-acting activators of homeotic genes

Kennison, Green, Cooper; in collaboration with Vázquez, Zurita

Genetic studies have identified the *trithorax* group of genes as required for expression or function of the homeotic genes. Reduced function of the *trithorax* group genes mimics loss of function of the homeotic genes. Many of the *trithorax* group genes have been shown to be required for the maintenance of transcription of the homeotic genes during development. Many trithorax group proteins are subunits of chromatin-remodeling or transcriptional coactivator complexes. We have identified at least two dozen *trithorax* group genes, most of which were not previously known. Two of the genes (*skuld* and *kohtalo*) encode subunits of the mediator coactivator complex. This complex is highly conserved between *Drosophila* and man, but only about a third of the subunits are conserved between yeast and man. We have identified several other trithorax group genes that encode subunits of chromatin-remodeling complexes; the *brahma*, *moira*, and *osa* genes encode subunits of the Brahma chromatin-remodeling complex, which is conserved from yeast (the SWI/SNF and RSC complexes) to man (the BRG1 and HBRM complexes). To understand further the function of the Brahma complex, we have been characterizing mutations that interact with mutations in the Brahma complex. For example, as part of these studies, we have isolated and characterized mutations in the *Asf1* histone chaperone as well as in the *Drosophila* homolog of the ELL RNA polymerase II transcriptional elongation factor [the *Su(Tpl)* gene]. We have recently identified two other genes (*taranis* and *tonalli*) that show genetic interactions with Brahma complex mutations. Both *taranis* and *tonalli* encode multiple protein isoforms. Some of the tonalli protein isoforms are particularly interesting, as they include an SP-RING finger domain that may function as a SUMO E3 ligase. SUMO is a small protein that is conjugated to target proteins to alter their function, cellular localization, or stability. The SUMO E3 ligases specify which target proteins get sumoylated. The genetic interactions with *tonalli* suggest that sumoylation may play an important role in regulating the function of the Brahma chromatin-remodeling complex.

Eissenberg JC, Ma J, Gerber MA, Christensen A, Kennison JA, Shilatifard A. dELL is an essential RNA polymerase II elongation factor with a general role in development. *Proc Natl Acad Sci USA* 2002;99: 9894-9899.

Gutiérrez L, Zurita M, Kennison JA, Vázquez M. The Drosophila trithorax group gene *tonalli* (*tna*) interacts genetically with the Brahma remodeling complex and encodes an SP-RING finger protein. *Development* 2003;130:343-354.

Moshkin YM, Armstrong JA, Maeda RK, Tamkun JW, Verrijzer CP, Kennison JA, Karch F. Histone chaperone ASF1 cooperates with the Brahma chromatin-remodelling machinery. *Genes Dev* 2002;16:2621-2626.

***Trans*-acting repressors of homeotic genes**

Kennison; in collaboration with Kassis

The initial domains of homeotic gene repression are set the by the segmentation proteins, which also divide the embryo into segments. Maintenance of repression requires the proteins encoded by the *Polycomb* group genes. We have identified a number of homeotic repressors, including the *Su(z)12*, *Mi-2*, and *Deaf-1* genes. To identify new *Polycomb* group repressors, we are screening for new mutations that either interact genetically with *Polycomb* mutations or mimic the homeotic phenotypes of *Polycomb* group mutations. We are conducting the screens in collaboration with Judy Kassis and the Section on Gene Expression, also within the Laboratory of Molecular Genetics.

Birve A, Sengupta AK, Beuchle D, Larsson J, Kennison JA, Rasmusson-Lestander A, Muller J. Su(z)12, a novel Drosophila Polycomb group gene that is conserved in vertebrates and plants. *Development* 2001;128: 3371-3379.

Veraksa A, Li X, Kennison J, McGinnis W. DEAF-1 function is essential for the early embryonic development of *Drosophila. Genesis* 2002;33:67-76.

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MOLECULAR GENETICS OF MAMMALIAN RETROVIRUS REPLICATION

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Our goal is to define the molecular mechanisms involved in the replication of HIV and related retroviruses. Our studies are critical for developing new strategies to combat the AIDS epidemic. To address these issues, we have developed reconstituted model systems to investigate the individual steps in HIV-1 reverse transcription, a major target of HIV therapy. Much of our work focuses on the viral nucleocapsid protein (NC), which promotes highly efficient and specific viral DNA synthesis. As a nucleic acid chaperone, NC can facilitate nucleic acid conformational rearrangements that lead to formation of the most thermodynamically stable structure. Such activity is essential for viral DNA synthesis. In other studies, our efforts are directed toward understanding the function of the viral capsid protein (CA) in HIV-1 assembly and early postentry events during the course of virus replication *in vivo*.

Role of nucleocapsid protein in HIV-1 strand transfer

Guo, Heilman-Miller, Levin; in collaboration with Gorelick, Henderson, Musier-Forsyth

HIV-1 NC is a small basic protein with two zinc fingers, each containing the invariant CCHC zinc-coordinating residues. NC function in virus replication is dependent on its dynamic interaction with nucleic acids the protein plays a critical role in the two strand transfer steps that occur during viral DNA synthesis. We have shown that, during minus-strand transfer, the nucleic acid chaperone activity of NC destabilizes the highly structured complementary TAR stem-loop (TAR DNA) at the 3′ end of (-) strong-stop DNA and inhibits TAR-induced self-priming, a dead-end reaction that competes with annealing of (-) strong-stop DNA to acceptor RNA, i.e., the strand transfer reaction. Mutational analysis has demonstrated that both NC zinc fingers are required for this function. In our recent work, we have focused on the influence of nucleic acid structure on NC nucleic acid chaperone activity and on measurement of NC-induced conformational changes in (-) strong-stop DNA, which form the basis for inhibition of self-priming.

To gain a better understanding of NC-mediated inhibition of self-priming, we have collaborated with Karin Musier-Forsyth and colleagues, who have developed a fluorescence resonance energy transfer (FRET) assay that makes it possible to monitor conformational changes directly in TAR DNA. The results show that, when NC binds to TAR DNA alone, only a modest shift occurs toward less folded conformations. In the presence of acceptor RNA, however, NC binding to TAR DNA results in a shift of the majority of molecules to the unfolded state. Interestingly, the FRET data are correlated with biochemical observations, indicating that self-priming is blocked only in the presence of acceptor RNA and NC, i.e., conditions that favor optimal minus-strand transfer. Taken together, the results support the possibility that NC-mediated annealing of nucleic acids is a concerted process involving a destabilization step that occurs in synchrony with hybridization.

In other work on nucleic acid chaperone activity, we are investigating the structural and thermodynamic requirements for NC interaction with strand transfer nucleic acid intermediates. We have constructed a series of synthetic (-) strong-stop DNA and acceptor RNA truncation mutants and are studying them by *in vitro* assay of minus-strand transfer and self-priming, enzymatic structure probing, and analysis of secondary structure using RNA and DNA structure prediction algorithms. As might be expected, truncations that disrupt the TAR DNA structure in (-) strong-stop DNA completely eliminate DNA self-priming. However, reducing or eliminating self-priming does not necessarily result in an increase in strand transfer efficiency; the structure of the acceptor RNA is also important. In fact, we have demonstrated that NC can mediate efficient strand transfer only when both (-) strong-stop DNA and acceptor RNA are moderately structured. Thus, little or no strand transfer occurs when the acceptor RNA is highly stable. The data demonstrate that a delicate thermodynamic balance between (-) strong-stop DNA and acceptor RNA must be maintained for efficient minus-strand transfer.

Studies are now in progress to determine the roles of NC and RNase H activity in facilitating the removal of short terminal RNA fragments that result from RNase H–catalyzed degradation of genomic RNA and that are initially annealed to (-) strong-stop DNA. The fragments must be removed to allow acceptor RNA to anneal to (-) strong-stop DNA so that elongation of minus-strand DNA can proceed. Preliminary results suggest that NC is efficient in facilitating fragment removal, even in the absence of RNase H.

Hong M, Harbron EJ, O'Connor DB, Guo J, Barbara PF, Levin JG, Musier-Forsyth K. Nucleic acid conformational changes essential for HIV-1 nucleocapsid protein-mediated inhibition of self-priming in minus-strand transfer. *J Mol Biol* 2003; 325:1-10.

Nucleic acid and protein requirements for initiation of HIV-1 reverse transcription

Iwatani, Levin; in collaboration with Gorelick, Musier-Forsyth

We have been investigating the initiation step in HIV-1 reverse transcription, an event primed by a host tRNA, $tRNA_3^{Lys}$, which is annealed to the 18-nt priming binding site (PBS) near the 5′ terminus of the viral RNA genome; extension of the primer leads to synthesis of the short DNA product known as (-) strong-stop DNA. In earlier work, we demonstrated that, in the absence of NC, the RNA template must contain a minimum of 24 bases downstream of the PBS when RNA primers ($tRNA_3^{Lys}$ or an 18-nt RNA complementary to the PBS [R18]), but not a DNA primer, are used. Surprisingly, when NC is present, the additional 24 bases downstream of the PBS are dispensable for synthesis primed by tRNA but not for synthesis primed by the R18 primer. We proposed that NC abrogates this requirement by facilitating stable formation of extended interactions between the full-length tRNA and the RNA template, which are not possible with an 18-nt RNA. Mutational analysis supports the possibility that NC facilitates an interaction between the 3′ arm of the anticodon stem and part of the variable loop of $tRNA_3^{Lys}$ and nt 143-149 in viral RNA. Collectively, the findings provide evidence that the nucleic acid chaperone activity of NC modulates the conformational stability of the initiation complex.

To analyze further the effect of NC on initiation, we tested by band-shift assay the affinity of reverse transcriptase (RT) for the viral RNA-tRNA complex in the presence or absence of NC. In the absence of dNTPs, NC does not affect RT binding to complexes constituted with either wild-type or mutant templates (substitution of the complementary bases in nt 143-149). In contrast, in a functional assay with a +1 extension of the tRNA primer, NC stimulates incorporation with the wild-type but not mutant templates. The results provide further evidence for a specific NC-dependent interaction outside the PBS region. In additional experiments conducted with low concentrations of dNTPs (5 microM), we find that NC significantly stimulates tRNA-primed (-) strong-stop DNA synthesis, even with a template with more than 24 downstream bases. Taken together, our data suggest that NC-facilitated interactions may also be important *in vivo*, where dNTP concentrations are thought to be relatively low and viral RNA is genome-size. Interestingly, studies with NC zinc finger mutants demonstrate that zinc coordination is not required for NC stimulation of (-) strong-stop DNA synthesis regardless of dNTP concentration. In our current work, we are performing enzymatic mapping studies to probe conformational differences in wild-type and mutant viral RNA-tRNA complexes in the presence and absence of NC.

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Iwatani Y, Rosen AE, Guo J, Musier-Forsyth K, Levin JG. Efficient initiation of HIV-1 reverse transcription *in vitro*: requirement for RNA sequences downstream of the primer binding site abrogated by nucleocapsid protein-dependent primer-template interactions. *J Biol Chem* 2003;278:14185-14195.

Functional analysis of HIV-2 reverse transcriptase activities

Post, Guo, Levin; in collaboration with Hizi, Le Grice, Powell

HIV-2 infection is a significant public health problem in West Africa and parts of Asia, yet there have been relatively few studies of HIV-2 replication as compared with the vast literature on HIV-1. Both viruses share similar genome organization, protein composition, and mode of transmission but differ in time of onset of AIDS and geographic pattern of infection. In addition, drugs that inhibit HIV-1 replication, e.g., nevirapine, are not necessarily effective against HIV-2, posing a major problem for clinical treatment of HIV-2–infected individuals. We have conducted a systematic evaluation of the functional activities of HIV-2 reverse transcriptase (RT), a potential target for antiviral therapy, using assays that model specific steps in reverse transcription. We conducted parallel studies with HIV-1 RT.

Under standard assay conditions, the RNase H and DNA- and RNA-dependent DNA polymerase activities of the two enzymes are comparable. However, when RT concentration is significantly reduced, HIV-2 RT is less active than the HIV-1 enzyme. HIV-2 RT is also compromised in its ability to catalyze secondary RNase H cleavages in assays that mimic tRNA removal from (-) DNA and 5′-directed RNase H activity (required for degradation of genomic RNA sequences as (-) DNA is synthesized). In addition, initiation of plus-strand DNA synthesis by the polypurine tract (PPT) primer is much less efficient with HIV-2 RT than with HIV-1 RT unless the salt and Mg^{2+} concentrations are drastically reduced. Previously, we showed that HIV-1 plus-strand initiation is dependent on nucleic acid contacts with "primer grip" residues in the palm subdomain of the p66 RT subunit. We have proposed that the reduced plus-strand priming activity of HIV-2 RT may reflect architectural differences in the primer grip regions in the two enzymes. We have also suggested that HIV-2 RT may have a lower binding affinity for the RNA PPT and other substrates used in our assays. Taken together, our findings should be useful for development of specific high-throughput screening assays of potential HIV-2 inhibitory agents.

Post K, Guo J, Howard KJ, Powell MD, Miller JT, Hizi A, Le Grice SFJ, Levin JG. Human immunodeficiency virus type 2 reverse transcriptase activity in model systems that mimic steps in reverse transcription. *J Virol* 2003;77:7623-7634.

Function of HIV-1 capsid protein in virus assembly and early postentry events

Tang, Levin; in collaboration with Freed

Our laboratory has been investigating the role of the HIV-1 capsid protein (CA) in early postentry events, a stage in the infectious process that is still not completely understood. Structural studies of CA showed that a group of conserved hydrophobic residues (including Trp23 and Phe40) faces the interior of the coiled coil–like structure within the N-terminal domain, and it was suggested that these residues might be important for CA structure and function. In our initial study, we used genetic, molecular, and ultrasound approaches and reported the unusual phenotype associated with single alanine substitution mutations in the residues. Mutant virions are not infectious and lack a cone-shaped core. Moreover, despite having a functional RT enzyme, the mutants are blocked in viral DNA synthesis in infected cells. The findings suggest that the mutations result in a defect in an early step preceding reverse transcription, which is correlated with a defect in assembly of viral cores.

Our recent work has focused on elucidating the mechanism by which the CA mutations disrupt virus infectivity. To investigate the possibility that the mutants might be compromised in an early postentry step, we modeled disassembly (i.e., viral uncoating) *in vitro* by generating viral cores from particles treated with mild detergent; cores were isolated by sedimentation in sucrose density gradients. In general, fractions containing mutant cores exhibit a normal protein profile. However, they demonstrate two striking differences from the wild-type pattern: mutant core fractions display a marked deficiency in RT protein and enzymatic activity (less than 5 percent of total RT in gradient fractions) and a substantial increase in the retention of CA. The high level of core-associated CA suggests that mutant cores may be unable to undergo proper disassembly. Taken together with the almost complete absence of RT in mutant cores, these findings can account for the failure of the three CA mutants to synthesize viral DNA following virus entry into cells.

We have also initiated studies to determine whether substitutions other than alanine result in a different phenotype; for this purpose, we constructed a series of vertical mutations in residues Trp23 and Phe40. We made a total of 13 additional substitutions at position 23 and two at position 40 (F40W and F40Y) as well as a double mutation, W23F/F40W. We tested the mutants in a single-cycle infectivity assay and observed that only mutant W23F exhibited infectivity, albeit at a very low level. W23F has a phenotype that appears to be intermediate between wild-type virus and the original W23A mutant. Interestingly, the W23F mutant (unlike W23A) is able to replicate during long-term culture in MT-4 cells, although we observed delayed replication kinetics. Our current efforts focus on isolating and characterizing second-site suppressors of the W23F mutation, which has the potential to yield important information about CA structure.

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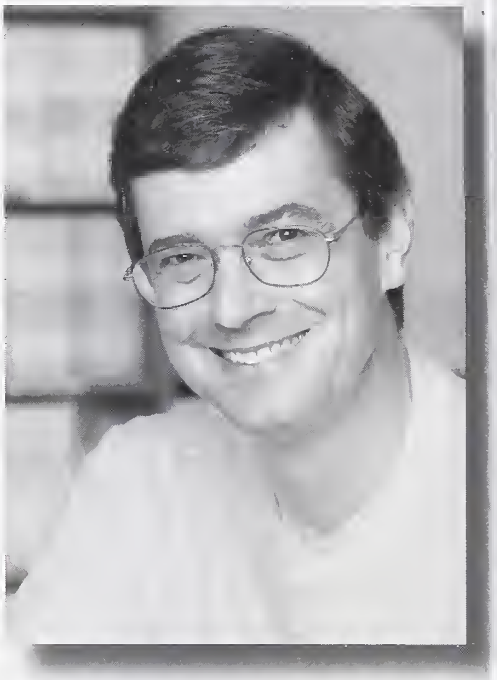
REGULATION OF ECTODERMAL DEVELOPMENT IN *XENOPUS*



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In vertebrate embryos, the ectoderm gives rise to the central nervous system, epidermis, sense organs, and neural crest. We are interested in the mechanisms that control this subdivision and differentiation process. In the frog *Xenopus,* the embryonic ectoderm can be easily isolated and induced to differentiate by experimentally activating or inactivating certain signaling pathways, such as bone morphogenetic proteins (BMP), Wnt factors, retinoids, and others. If left undisturbed, the ectoderm differentiates as epidermis. We use this model system to identify and test the function of regulatory proteins, primarily transcription factors that mediate the transition from the epidermal to other programs. Our current focus is on proteins encoded by a family of homeobox genes, the Dlx and Msx factors, and on the transcriptional activator factor AP2alpha. We have shown that the *Dlx/Msx* genes play important roles in the early development of the inner ear and the neural crest and that AP2alpha function is essential for both epidermis and neural crest. We are now using *Xenopus* microarrays to identify the transcriptional target genes for AP2alpha. In addition to already characterized genes, we have found approximately 100 novel targets for AP2alpha, which are currently under investigation.

Dlx and *Msx* in ectodermal patterning and development

Luo, Khadka, Zhang, Sargent; in collaboration with Saint-Jeannet

Vertebrate species have roughly six homologs of the *Drosophila* homeobox gene *Distal-less* (*Dlx1-Dlx6*) and three of *Muscle-specific* homeobox, or *Msh* (*Msx1-Msx3*). The Dlx factors are transcriptional activators, and the Msx factors are repressors, with both families binding to essentially identical DNA sequences. *Dlx* and *Msx* genes tend to be expressed in adjacent and overlapping tissues that are undergoing transitions from proliferation to differentiation and/or interacting with one another. These factors have been implicated in the development of many tissues, including limbs, epidermis, hair, teeth, and bones. We found that, in the early gastrula stage of *Xenopus*, Dlx3, Dlx5, Dlx6, and Msx1 are expressed in overlapping territories in the ectoderm and that this pattern seems to be controlled largely by the degree to which the individual genes depend on the local strength of a morphogenetic signal based on BMPs and BMP antagonists. When this pattern was disrupted by ectopically expressing Dlx and Msx factors, specific sets of locally expressed genes were inactivated. For example, forcing expression of *Msx1* in the cement gland tissue where it is normally silent results in inhibition of all cement gland genes. The same treatment with Dlx factors, which are normally expressed in cement gland, has no effect. Similarly, ectopic expression of *Dlx3* in neural crest eliminates this tissue type. Based on these observations, we proposed a model in which *Dlx/Msx* genes "read" the BMP signal gradient and define ectodermal cell types, mainly by inhibitory mechanisms.

The model predicts that loss of *Dlx/Msx* gene function should lead to expansion of the inhibited territories. To test the model, we have used antisense morpholino oligonucleotides (MOs) that block translation or RNA splicing in a gene-specific manner. So far, we have analyzed Dlx3 and Msx1 functions in this way but did not observe the predicted expansions, thus requiring us to modify or discard the model. For example, it is possible that Dlx/Msx factors function in a "permissive" mode and that removing them does not result in expanded domains unless "instructive" signals are also increased. Alternatively, functional redundancy may mask some loss-of-function phenotypes. Given that such loss often occurs when genes regulating early pattern formation are knocked out in mouse, the phenomenon is not surprising. On the other hand, the MOs did result in some interesting effects: loss of Dlx3 resulted in the partial silencing of an important regulatory factor, Sox9, in the presumptive inner ear while loss of Msx1 function resulted in the inactivation of some, but not all, neural crest regulatory genes and subsequent major deficiencies in neural crest derivative tissues, particularly in the head.

The *Dlx5* and *Dlx6* genes are strongly active in the band of tissue that gives rise to the sensory placodes, suggesting that these genes are also important, along with *Dlx3*. By designing MOs to inhibit Dlx5 and Dlx6, we will investigate the importance of *Dlx5* and *Dlx6*. Eventually, combinatorial inhibition of *Msx* and *Dlx* genes should help clarify the roles that the encoded factors play in ectodermal development. Given that these genes are all expressed in similar patterns in mammalian embryos, including humans, the results of these studies should have important medical and developmental implications.

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AP2alpha and induction of epidermis and neural crest

Sargent, Luo; in collaboration with Cho, Saint-Jeannet

AP2alpha is a transcriptional activator protein that was identified several years ago, one of the first such proteins to be cloned and characterized. Our laboratory originally identified AP2alpha as a potential regulator of a keratin gene expressed very early in epidermal development. Using antisense oligonucleotides, dominant negative mutant proteins, and ectopic expression strategies, we showed that AP2alpha is indeed a direct activator of the keratin gene and controls a large spectrum of other genes in skin. More recently, we have investigated the role of this factor in neural crest, where it is strongly expressed. In *Xenopus*, and probably in other vertebrates, neural crest induction requires two signaling inputs: BMP, which must be partially attenuated, and a Wnt/beta catenin signal. We have found that AP2alpha is the factor that mediates the BMP portion of the inducing signal. Using a form of AP2alpha that can be activated by treating embryos with dexamethasone, we can, under appropriate experimental conditions, turn on most if not all of the neural crest program in isolated ectoderm simply by adding the drug to the saline solution in which the ectoderm is incubated. Some neural crest genes that are activated by AP2alpha in this way appear to be direct targets, as the activation is not blocked by earlier treatment with protein synthesis inhibitors. In one case, the *Xenopus slug* gene, we have located what appear to be functional AP2alpha binding sites in the 5′ flanking region. We have used the same procedure to produce fluorescently labeled probes for screening *Xenopus* microarrays and have identified by this means approximately 100 new AP2alpha targets, which are currently under study.

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ORGANOGENESIS OF THE ZEBRAFISH VASCULATURE

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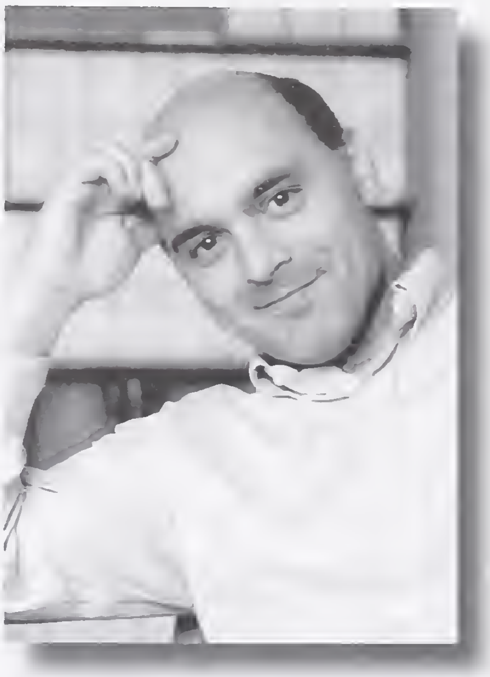

The overall objective of our project is to understand the cellular and molecular mechanisms underlying organogenesis, in particular how the elaborate network of blood vessels arises during vertebrate embryogenesis. Blood vessels are ubiquitous and vital components of all vertebrate animals, innervating and supplying every tissue and organ with oxygen, nutrients, and cellular and humoral factors. They have become a subject of great clinical interest in recent years due in large part to the potential shown by antiangiogenic therapies for combating cancer. The zebrafish, a small tropical freshwater fish, possesses a unique combination of features that make it particularly well suited for studying blood vessel formation (Weinstein, 2002). The fish is a genetically tractable vertebrate with a physically accessible, optically clear embryo that permits easy observation of every vessel in the living animal and simple, rapid screening for even subtle vascular-specific mutants. We aim to develop experimental tools and resources to enhance the zebrafish as an experimental model for studying vascular embryogenesis, investigating the molecular basis for arterial-venous differentiation, exploring the basis for vascular patterning and morphogenesis, and identifying, by forward-genetic analysis, novel zebrafish genes critical for vascular development.

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Tools for experimental analysis of vascular development in the zebrafish

Bae, Isogai, Kamei, Kidd, Lawson, Torres-Vazquez; in collaboration with Davis, Johnson

To develop new experimental tools for studying blood vessel formation in the zebrafish, we previously established a microangiographic method for imaging patent zebrafish blood vessels and used it to compile a comprehensive staged atlas of the vascular anatomy of the developing fish. We also generated a number of different transgenic zebrafish lines expressing green fluorescent protein (GFP) in vascular endothelial cells (VEC), making it possible to visualize blood vessel formation in intact, living embryos (Lawson and Weinstein, 2002). We have developed methodologies for long-term multiphoton confocal time-lapse imaging of the dynamics of blood vessel formation in these transgenic fish and have used these methodologies to examine the morphogenesis of developing trunk (Isogai *et al.*, in press; see "Analysis of vascular patterning and morphogenesis" below), cranial vessels (Lawson and Weinstein, 2002), and adult fin blood vessels (Huang *et al.*, in press). Our findings highlight the highly dynamic and unexpectedly growth cone–like behavior of growing angiogenic blood vessels and are reinforced by our analysis of the role of growth cone guidance factors in vascular patterning (see below). One of our current priorities is the development of additional transgenic lines that permit dynamic visualization of specific subsets of vessels such as arteries and veins or subcellular structures within vascular cells (see below) or that allow temporally regulated gene expression within the vasculature. We are also continuing to acquire and characterize new vascular-specific zebrafish genes and to develop antibody reagents for assaying their gene products (in our experience, antibodies directed against mammalian proteins generally do not cross-react well with zebrafish proteins; we have so far generated antibodies recognizing zebrafish flk1, VE-cadherin, and plexinD1 and are in the process of preparing antibodies against additional vascular genes).

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Molecular dissection of arterial-venous development

Bae, Kidd, Lawson, Mugford; in collaboration with Chitnis, Hoying

We have detailed a molecular pathway regulating the acquisition of arterial-venous identity. Although the fundamental distinction between these two types of blood vessels has been appreciated for thousands of years, the fact that arterial and venous endothelial cells have distinct molecular and functional identities has only recently become apparent, and the mechanisms responsible for establishing such identity have not been elucidated. We have now shown that sonic hedgehog (SHH), vascular endothelial growth factor (VEGF), and notch signaling act in series to determine arterial-venous identity (Lawson *et al.*, 2002). Accordingly, we used different combinations of drug treatments, mutants, morpholinos, and mRNA injections to activate or repress the activity of each of these signals *in vivo,* either alone or in combination. Subsequent studies from other laboratories have supported a similar role for this novel pathway during mammalian arterial differentiation. Using genetic screening and positional/candidate cloning methods, we recently identified *y10,* a mutant in *phospholipase C gamma-1*, and demonstrated that the gene is a major downstream effector of VEGF signaling *in vivo* (Lawson *et al.*, 2003). We are continuing to identify potential mutants in genes in this pathway in our ongoing genetic screen. We have also recently begun microarray screening by using the available genetic and experimental tools to activate or inhibit arterial differentiation at different steps. With these two complementary approaches, we will continue to fill in the pathway we have elucidated with additional genes.

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Analysis of vascular patterning and morphogenesis

Berk, Isogai, Lawson, Torres-Vazquez; in collaboration with Chien, Childs, Davis, Epstein, Fishman, Li

We have begun to study the mechanisms and molecular basis for vascular patterning and morphogenesis during development, in particular how vascular networks assemble with a defined, stereotypic anatomical pattern and how vessels undergo lumen formation. We have used multiphoton time-lapse imaging of the developing trunk to obtain important new insights into how angiogenic networks assemble *in vivo*, including a novel two-step model for vascular network formation and evidence for genetic determination of vascular pattern (Isogai *et al.*, in press). We have also defined some of these genetic pattern determination factors, showing that well-known neuronal guidance factors also play an important role in vascular guidance and vascular patterning. Slit-Robo and semaphorin-plexin ligand-receptor pairs play important roles in axonal pathfinding, mostly via repulsive interactions between Slit or semaphorin ligands and Robo- or plexin-bearing neuronal growth cones. In collaboration with researchers studying related murine genes, we have uncovered novel Robo and plexin receptors expressed in zebrafish blood vessels and examined their functional roles. Our analysis of the role of semaphorin-plexin signaling has shown that it is an essential determinant of trunk vessel pattern. The *plxnD1* gene is expressed in an entirely vascular-specific fashion in both mice and zebrafish. In the developing trunk, angiogenic intersegmental vessels extend near somite boundaries. Loss of *plxnD1* function via morpholino injection or in the zebrafish *out of bounds (obd)* mutant causes dramatic mispatterning of these vessels, which are no longer restricted to growth near intersomitic boundaries. Somites flanking intersegmental vessels express semaphorins, and reducing the function of these semaphorins also causes intersegmental vessel mispatterning. The results indicate that the establishment of anatomical pattern in the developing vasculature is directed in part by cues and mechanisms similar to those used to pattern the developing nervous system, including semaphorin-plexin signaling. We are currently attempting to extend these studies and uncover the signal transduction machinery that transmits semaphorin-plexin signaling. Using genetic screening methods, we are also identifying additional loci that influence vascular patterning *in vivo.*

In separate studies, we have generated a transgenic line that should aid us in visualizing the process of tubular morphogenesis during vessel formation *in vivo*. A variety of studies have suggested that vessels may form lumens via intra- and intercellular fusion of large vacuolar organelles within angioblasts and endothelial cells, but definitive *in vivo* evidence for this idea has been lacking. Using high-sensitivity multiphoton time-lapse imaging of a transgenic line that preferentially highlights these organelles, we are attempting to provide the first dynamic *in vivo* look at how lumen formation occurs.

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Genetic analysis of vascular development

Bae, Berk, Kamei, Kidd, Lawson, McElwain, Mugford, Pham, Roman, Torres-Vazquez; in collaboration with Childs, Dawid, Liu, Moon, Stainier

To dissect the genetics of vascular development and the molecular pathways that regulate it, we employ unbiased, forward-genetic mutational screening approaches that allow us to identify and then phenotypically and molecularly characterize zebrafish mutants that affect the formation of the developing vasculature. We have positionally cloned the defective genes from a number of different vascular patterning mutants. As a result of defects in a zebrafish ortholog of the TGF-beta superfamily receptor *acvrl1* (Roman *et al*., 2002), *violet beauregarde* mutants have abnormal cranial vascular patterning and circulation. Defects in human *acvrl1* cause hereditary hemorrhagic telangiectasia type 2 (HHT), an inherited vascular disorder characterized by arterial-venous malformations with a high incidence of hemorrhage and stroke. As noted above, *y10* mutants have defects in the *plcg1* gene (Lawson *et al*., 2003, see above). Another mutation, *kurzschluss,* has defects in the posterior aortic arches caused by defects in *smap1*, which encodes a chaperonin that is expressed in the mesenchyme surrounding the arch vessels and that may be involved in regulating myosin assembly. In collaborative studies, we have also determined the molecular basis for *silent heart,* a defect in *cardiac troponin T,* that results in a morphologically normal but nonbeating heart (Sehnert *et al*., 2002); *vlad tepes,* a defect in *gata1* that causes a bloodless phenotype (Lyons *et al*., 2002); and *out of bounds* (see above), a mutant in *plxnD1*. Molecular characterization of additional mutants obtained in ongoing screens is in progress.

We have successfully performed screens for new vascular-specific mutants by using transgenic zebrafish expressing green fluorescent protein (GPF) in blood vessels. We identified 11 new vascular-specific mutants in a pilot screen (approximately 500 genomes screened) of haploid progeny of ENU-mutagenized animals that was performed with the Dawid and Liu laboratories (members of the laboratories independently screened for hematopoietic and neuronal defects), identifying mutants in *plcg1, flk1,* and several additional as yet undetermined genes. In collaboration with members of the Dawid laboratory, a larger-scale F3 diploid screen of ENU-mutagenized animals is now in progress (currently up to approximately 700 mutagenized genomes) to screen for mutants affecting both intersegmental vessel formation and later vascular patterning events that cannot be examined in haploid animals. To date, our F3 screen has yielded an additional 15 vascular-specific mutants with phenotypes that include loss of most vessels or subsets of vessels, increased sprouting/branching, and vessel mispatterning. Molecular characterization of these mutants is in progress. Our experience so far suggests that ongoing mutant screens should continue to yield a rich harvest of novel vascular-specific mutants and bring to light new pathways regulating the specification, differentiation, and patterning of the developing vertebrate vasculature.

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MECHANISMS FOR CONTROLLING GENE EXPRESSION IN BACTERIA AND BACTERIOPHAGE

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Temperate bacteriophages can establish lysogeny, a long-term and mutually beneficial association with their bacterial hosts. Lysogeny is a major mechanism of horizontal gene transfer between bacterial families. Temperate phages constitute a reservoir of genes, such as those encoding virulence factors, that allow their hosts to survive environmental changes. To establish and maintain lysogeny, an infecting phage must carefully control the expression of its own genes so that host viability is unimpaired. It must also synchronize its own replication with that of the host. We report here the mechanisms that some phages use to resolve each of these problems.

Mechanism and control of transcription termination

King, Komissarova, Read, Rutkai, Sen, Sloan, Velikodvorskaya

The ability of cells to adjust to environmental stress and choose between alternative pathways of development requires precise control of gene expression. RNA polymerase (RNAP), a large, multisubunit enzyme whose core is structurally and functionally conserved in all kingdoms of life, catalyzes transcription, the first step in gene expression. After initiating transcription, RNAP continues to elongate the transcript until it reaches a terminator site, where elongation stops, and the enzyme, template, and transcript dissociate. Terminators insulate contiguous groups of genes from each other, thereby allowing independent control of the transcription of each group. In addition, the efficiency of termination and hence the expression of downstream genes can be controlled by antiterminators. Terminators help to silence virus gene expression in lysogens, and antiterminators allow expression of these genes during normal virus growth. The molecular mechanisms of both processes are poorly understood. We study an antitermination mechanism found in phage HK022, a virus that parasitizes the bacterium *E. coli*.

HK022 uses a novel, RNA-based antitermination mechanism to express many essential genes. A nascent transcript of a viral sequence called *put* binds to the RNAP molecule that catalyzed its synthesis and remains associated with it as the enzyme elongates the transcript. Association with *put* RNA modifies RNAP so that it resists termination at downstream terminators. No other protein factor is required, other enzyme molecules in the same cell are unaffected, and there is no apparent terminator specificity. The elongation control pathway appears simpler than others, and its simplicity makes it an attractive target for deeper analysis. We wish to determine how interaction with *put* RNA changes the properties of RNAP.

Characterization of RNAP mutants that specifically block *put* antitermination helped identify sites of interaction between *put* RNA and the elongation complex. The mutations alter a zinc binding domain (the Zn finger), a surface-exposed loop of the beta′ subunit. The tip of the Zn finger contains two pairs of invariant cysteines that ligate a zinc atom. The cysteines flank a moderately well conserved segment of 13 amino acids that is rich in basic residues. Replacement of individual residues or deletion of the fingertip decreased *put*-dependent antitermination. Some mutants, including the deletion, were unable to antiterminate, but others retained residual activity. In some cases, the residual activity could be greatly increased by a change in the *put* sequence. Such allele specificity suggests that the fingertip interacts directly with *put* RNA, a notion supported by the observation that a completely defective mutant was unable to bind to *put* RNA. Surprisingly, a larger deletion that removed the entire finger partially restored antitermination. Our working model is that *put* RNA interacts with the zinc finger and then with a second site in RNAP. Interaction with the zinc finger opens access to the second site, but amputation of the zinc finger obviates the need for the initial interaction by unmasking the second site. The location of the second site is unknown.

We suggest that *put* reduces termination by increasing the elongation rate. The elongation complex pauses at sequences that are rich in U residues, and such U-rich sequences are essential components of "intrinsic" terminators. *Put* suppresses such pausing, perhaps by inhibiting RNAP "backtracking." When the elongation complex is halted at the end of a U-rich sequence, it moves backwards on the template with consequent disengagement of the 3′ RNA end from the active site and concerted retreat of the RNA:DNA hybrid region from the 3′ end. Backtracking removes the 3′ end of the nascent transcript from the active site and exposes upstream phosphodiester bonds to hydrolysis activated by the GreB protein. It also delays resumption of elongation until the 3′ end is restored to the active site by forward sliding. We found that the beta′-Y75N mutation, which prevents *put* RNA binding, enhanced GreB cleavage and delayed resumption of elongation when RNAP was artificially arrested at a U-rich pause site by a Lac repressor roadblock and then restarted by the addition of IPTG. We propose that *put* RNA antiterminates by favoring the posttranslocational state of the elongation complex, a state in which the catalytic site of the enzyme is engaged with the next incoming templated substrate.

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Evolution of insertion site specificity

Rutkai, Dorgai, Yagil

Many temperate bacteriophages lysogenize their hosts by inserting their chromosomes into *attB*, a specific attachment site in the host chromosome. The inserted phage chromosome replicates coordinately with that of the host. Insertion requires integrase, a phage-encoded protein. Integrase catalyzes DNA strand exchange between *attB* and an attachment site in the phage chromosome (*attP*). Bacterial chromosomes contain many *attB*s, each specific to one or a few phages. Although different integrases share a common catalytic mechanism and are related to each other in structure, those encoded by different phages typically recombine only their cognate sets of attachment sites. The existing relationships and distribution of integrases and *attB*s imply that phages occasionally change their insertion specificity over an evolutionary time scale. Alteration of insertion specificity cannot occur in a single step because it requires changes of both integrase and the multiple integrase

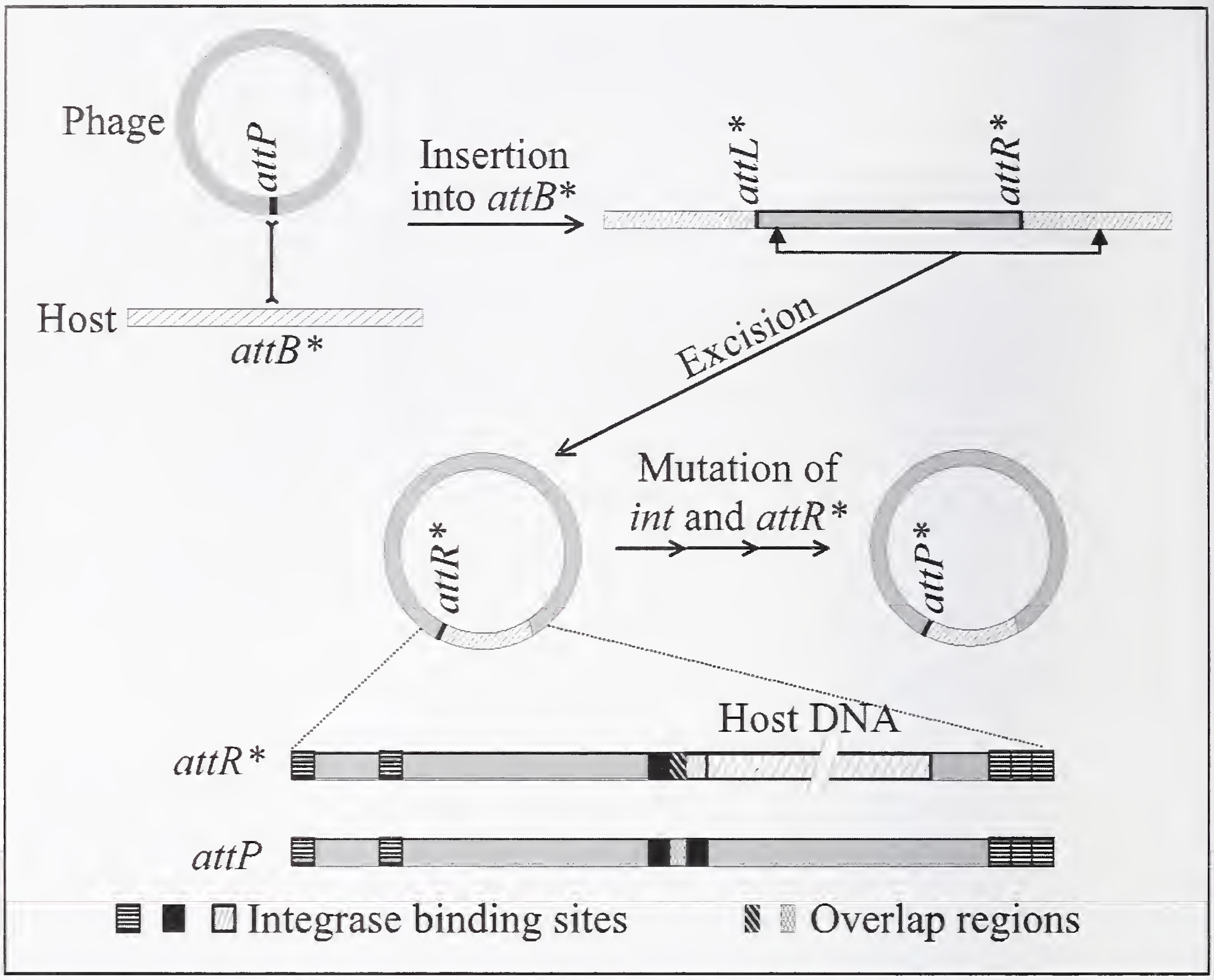

FIGURE 17.1 How Changes in Bacteriophage Insertion Secificity Evolve

First, a phage inserts its chromosome into a secondary attachment site with a variant overlap region (*attB**). For phage Lambda, we have shown that the overlap region of *attB** nearly always segregates into *attR**, the attachment site at the right of the inserted phage chromosome. Then, excision at the points indicated by the arrowheads produces a phage chromosome with *attR** in place of *attP*. *AttR** contains the variant overlap region and all but one of the integrase binding sites of wild-type *attP* but lacks full insertion activity at *attB** because one of the integrase binding sites is not wild type and because of the potentially large substitution of host for phage DNA adjacent to the overlap region. Mutations of *attR**, for example, a partial deletion of the substituted host DNA, will increase insertion activity, and mutations of integrase will increase utilization of the integrase binding sites of *attB**. The model has the attractive feature that new *attP* and *attB* sites evolve from sequences that already have activity as integrase substrates, thereby allowing alteration of insertion specificity by a series of relatively small steps that limit loss of insertion capacity. In addition, the *attP* sites of four known phages have the structure predicted by the model: an overlap region at one end of a segment of DNA that is clearly of host origin. In other phages, such as HK022 and Lambda, sequence that could be of host origin is small, possibly because most of the more extensive initial host substitution has been deleted.

binding regions within the attachment sites. In addition, efficient insertion requires sequence identity within the "overlap regions" of the recombining sites. The overlap region is a 7 bp DNA segment that lies between the points of DNA strand exchange, and different phages typically have different overlap regions. Finally, the types and order of mutations that must occur are constrained by the condition that insertion-defective phages are at a selective disadvantage.

We are using two sets of closely related but functionally distinct integrases and *att*s, those of phages Lambda and HK022, as tools to understand how evolutionary changes in insertion specificity might occur. We have shown that the recombination specificity of Lambda integrase can be changed to that of phage HK022 by a relatively small number of amino acid replacements. Some changes relax specificity: they allow the enzyme to recombine new sites without compromising recombination of the old. Others restrict specificity: they prevent the enzyme from recombining the original sites. However, changes in *attP* are also needed to change insertion specificity, and it is likely that integrase and *attP* coevolve. We propose that phage insertion into secondary attachment sites contributes to alteration of *attP* specificity. Insertion of phage Lambda into secondary sites is catalyzed by integrase and occurs many orders of magnitude more frequently than mutation. The integrase binding sites and overlap regions of secondary sites are similar but not identical to those of *attB*. We characterized the most frequently used secondary sites and measured how often they are used by wild-type phage and by those carrying relaxed, restricted, or both types of integrase mutations. We also characterized the products of insertion at secondary sites whose overlap region differs from that of the primary. We have incorporated the findings into a model for evolutionary change in insertion specificity (see Figure 17.1).

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GENE ACTIVATION IN YEAST: TARGETED HISTONE ACETYLATION AND REMODELED CHROMATIN DOMAINS

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Gene activation involves the ordered recruitment of factors to a promoter in response to appropriate signals, ultimately resulting in the formation of an initiation complex by RNA polymerase II and subsequent transcript elongation. These events must occur in the presence of nucleosomes, which are compact structures capable of blocking transcription at every step. To circumvent the chromatin block, eukaryotic cells possess a set of chromatin-remodeling and nucleosome-modifying complexes. The former (e.g., the SWI/SNF complex) use ATP to drive conformational changes in nucleosomes and to move nucleosomes along DNA; the latter contain enzymatic activities (e.g., histone acetylases) that modify the histones post-translationally to mark them for recognition by other complexes. Geneticists have described many interesting connections between chromatin components and transcription, but a system to investigate the structural basis of these connections has been lacking. We have developed such a model system, involving native plasmid chromatin purified from the yeast *Saccharomyces cerevisiae*, to perform high-resolution studies of the chromatin structures of active and inactive genes. Remarkably, they reveal that activation correlates with large-scale movements of nucleosomes and conformational changes within nucleosomes over entire genes. Our current work focuses on understanding the role of this extensive remodeling in transcription.

Remodeling and acetylation at CUP1

Akhavan, Eriksson, Naim, Shen

We chose budding yeast as a model organism because it permits biochemical studies of chromatin structure combined with molecular genetics. In particular, we selected the *CUP1* gene, as its regulation is well understood: *CUP1* encodes a metallothionein required to protect cells from the toxic effects of copper in the medium. Copper ions enter the cell and bind to the N-terminal domain of a transcriptional activator Ace1p, resulting in the folding of this protein to form a DNA-binding domain that recognizes upstream activating sequences in the *CUP1* promoter.

We purified plasmid chromatin containing the *CUP1* gene from copper-induced or uninduced cells. Using the monomer extension method, we determined the changes occurring in the chromatin structure of *CUP1* on activation by mapping nucleosome positions at high resolution. Our work revealed that induction results in the Ace1p-dependent movement (sliding) of nucleosomes over the entire *CUP1* gene, not just at the promoter. Transcription is not responsible for the movement because the TATA boxes are not required. We propose that a chromatin-remodeling activity capable of sliding nucleosomes along *CUP1* DNA is recruited by Ace1p to *CUP1*. Our observations provide direct evidence *in vivo* for nucleosome movement as a result of *CUP1* induction and indicate that remodeling is not restricted to the promoter but rather occurs over a chromatin domain defined by *CUP1* and its flanking sequences (Shen *et al.*, 2001).

Using purified core histones and DNA, we investigated the role of DNA sequence in determining nucleosome positions on *CUP1 in vivo* by comparing the positions observed in native chromatin with the positions adopted by nucleosomes reconstituted on *CUP1 in vitro*. We mapped multiple positions, almost all identical to those observed *in vivo*. DNA sequence therefore plays an important role in positioning nucleosomes *in vivo* because it contains information specifying many overlapping positions. Remodeling machines appear to use this information when moving nucleosomes around on *CUP1 in vivo* (Shen and Clark, 2001).

Induction of *CUP1* results in targeted acetylation of both histones H3 and H4 at the *CUP1* promoter. Targeted acetylation requires Ace1p and the TATA boxes, implying that acetylation occurs when TBP binds to the TATA boxes or at a later stage in initiation. Given that current models suggest that the function of targeted acetylation is to facilitate the binding of TBP to TATA boxes in nucleosomes, our result proved surprising and is a major focus of our current work. By testing various HAT mutants for growth defects in copper, we identified the histone acetyltransferase (HAT) activity responsible for targeted acetylation at *CUP1*. We found that disruption of the putative HAT encoded by the *SPT10* gene is lethal at high copper concentrations and correlates with impaired *CUP1* induction and loss of targeted acetylation. Others have identified *SPT10* as a global regulator of core promoter activity, which fits well with the TATA box requirement for acetylation at *CUP1*. We suggest that acetylation might facilitate the binding of Ace1p to nucleosomal binding sites (Shen *et al.*, 2002).

Our current work focuses on (1) identifying the remodeling complex responsible for nucleosome sliding on *CUP1* by testing various remodeling mutants for growth defects in copper and (2) determining the order of recruitment of factors at the *CUP1* promoter after copper induction by using chromatin immunoprecipitation (ChIP) experiments (proteins of interest include Ace1p, TBP, Spt10p, and RNA polymerase II), and (3) elucidating the role of the TATA boxes in targeted acetylation of nucleosomes at the *CUP1* promoter. We have evidence that Spt10p associates with the *CUP1* promoter in a copper-dependent fashion. We are assessing the effects of mutations in the HAT domain of Spt10p and in the zinc finger domain (a putative DNA-binding domain) on the recruitment to and activity of *CUP1*. We shall characterize the HAT activity of Spt10p by using both recombinant Spt10p and Spt10p from yeast. It is likely that the latter takes the form of a multisubunit complex; we shall identify the other subunits by mass spectroscopy. Taken together, this information should allow us to build a highly detailed picture of how *CUP1* chromatin is activated for transcription.

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The SWI/SNF complex and remodeling of *HIS3*

Kim

Current models for the role of the SWI/SNF ATP-dependent chromatin remodeling complex in gene regulation focus on promoters, which is where the most obvious changes in chromatin structure occur. However, by using our plasmid model system with *HIS3*, an SWI/SNF-regulated gene, we discovered that induction of *HIS3* creates a domain of remodeled chromatin structure that extends far beyond the promoter to include the entire gene. Induction results in a dramatic loss of nucleosomal supercoiling, a decompaction of the chromatin, and a general increase in the accessibility of the chromatin to restriction enzymes. Formation of the domain requires the SWI/SNF complex and the activator Gcn4p, but not the TATA boxes, indicating that remodeling does not result from transcription. The implication is that the nucleosomes have been opened up in a SWI/SNF-dependent remodeling reaction. We propose that the SWI/SNF complex is recruited to the *HIS3* promoter by Gcn4p and then directs remodeling of a chromatin domain, perhaps facilitating transcription through nucleosomes in keeping with a possible role for the SWI/SNF complex as an elongation factor (Kim and Clark, 2002).

Elucidation of the structure of the remodeled nucleosome involves two possibilities: unstable nucleosomes (remodeled such that they fall apart easily) and nucleosomes with a dramatically altered conformation. To distinguish between the nucleosomes, we shall measure the core histone: DNA stoichiometry in uninduced and induced plasmid chromatin, which should reveal any loss of histones. Based on our current observations, the latter possibility is more likely. To test our hypothesis, we shall probe the accessibility of the single cysteine sulphydryl group of H3, which is normally buried within the nucleosome and protected from sulphydryl-reactive reagents. If the group proves accessible, the implication is that the H3/H4 tetramer, which constitutes the kernel of the nucleosome, has been opened up. We shall use ChIP to determine whether a correlation occurs with the presence of histone variants, such as H2A.Z. Given that targeted acetylation occurs at the *HIS3* promoter, determination of the roles of histone modifications in remodeling requires an investigation of HATs Gcn5p and Esa1p by using the appropriate mutants. We shall also address the roles of histone methylation, phosphorylation, and ubiquitination. Given that targeted acetylation occurs at the *HIS3* promoter, determination of the roles of histone modifications in remodeling requires an investigation of HATs Gcn5p and Esa1p by using the appropriate mutants. We shall also address the roles of histone methylation, phosphorylation, and ubiquitination.

An elucidation of the role of the Isw1p remodeling complex will follow from our analysis of nucleosome positioning on *HIS3,* which established that, as for *CUP1*, activation results in the disruption of an ordered nucleosomal array and the sliding of nucleosomes into the linker regions. At *HIS3*, the SWI/SNF complex is required for nearly all the nucleosome mobilization. We have found that Isw1p is also required for remodeling nucleosome structure and therefore are currently focusing on understanding how the two remodeling machines cooperate to activate *HIS3*. Making comparisons with chromosomal HIS3 is necessary to confirm our experiments performed with plasmid-borne *HIS3*. To that end, we shall construct an inducible *FLP* recombinase system to study a strain with a chromosomal *HIS3* gene that can be excised to form a plasmid. We shall use nucleases to probe the chromatin structure of *HIS3* and shall examine the topology after excision of the *HIS3* gene to generate a plasmid derived from the chromosome.

Our work on *CUP1* and *HIS3* indicates that, at least for these two genes, the target of remodeling complexes is a domain rather than just the promoter. This finding is important because it suggests that remodeling complexes act on chromatin domains. The question arises as to the function of domain remodeling. We speculate that remodeling entire genes might facilitate elongation through nucleosomes by RNA polymerase II. In a wider context, the fact that remodeling complexes can participate in the formation of chromatin domains might be important in understanding the formation of domains in higher eukaryotes (discussed in Oliver *et al*., 2002).

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RNA polymerase II phosphorylation and transcription through nucleosomes

Clark; in collaboration with Studitsky

Carried out in collaboration with Vasily Studitsky, our studies continue our earlier work aimed at deciphering the mechanism of transcription through the nucleosome. Although RNA polymerase II is able to transcribe through a nucleosome *in vitro*, it does so at an exceedingly slow pace, far slower than the measured rates of transcription through chromatin *in vivo*. As a result, several investigators have attempted to identify factors that might dramatically improve the transcription rate through the nucleosome *in vitro*. One possibility is a role for the phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II. CTD phosphorylation occurs *in vivo* as RNA polymerase II is converted from its initiating form to the elongation complex. It is therefore the phosphorylated form of the polymerase that is responsible for transcription through nucleosomes *in vivo*. In this work, we compared the rates of transcription through the nucleosome by phosphorylated and unphosphorylated polymerase. However, CTD phosphorylation had little effect on the rate. Thus, if CTD phosphorylation has a role in transcription through the nucleosome *in vivo*, it must be indirect (Liu *et al*., 2003).

Liu Y, Clark DJ, Tchernajenko V, Dahmus ME, Studitsky VM. Role of CTD phosphorylation in transcription through the nucleosome by RNA polymerase II. *Biopolymers* 2003;68:528-538.

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REGULATION OF DNA REPLICATION AND GENE EXPRESSION DURING ANIMAL DEVELOPMENT

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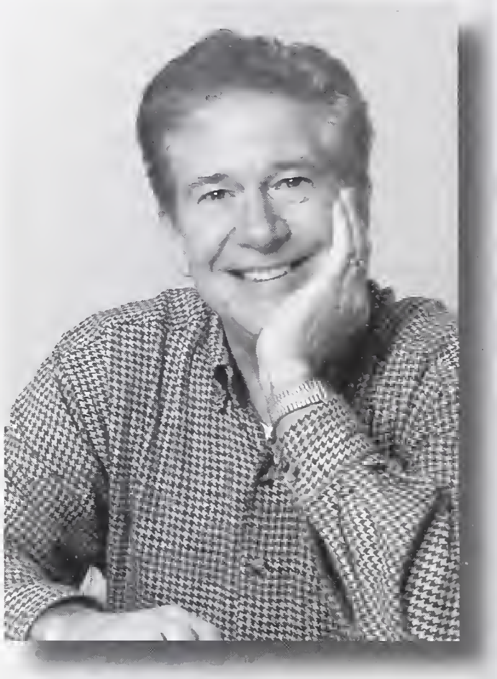

In the field of eukaryotic DNA replication, we have focused on how the metazoa regulate the number and locations of initiation sites during cell proliferation and development. In previous studies, we mapped origins of bidirectional replication (OBRs) to specific chromosomal sites of 0.4 to 2 kb in mammalian cells, demonstrated that prereplication complexes are assembled at or close to these sites when cells transit from metaphase to G1 phase during cell division, and discovered that one of the six subunits (Orc1) of the "origin recognition complex" (ORC), which is responsible for determining where replication begins, is selectively released from chromatin before or during metaphase and then rebound to chromatin during the M to G1 transition, resulting in the appearance of prereplication complexes at specific genomic sites. Our research suggested that, by preventing assembly of prereplication complexes until mitosis is complete and a nuclear membrane has reformed, ORC determines not only where but also when replication begins. Such an "ORC cycle" would represent a novel mechanism for regulating cell division and the premier step in regulating the onset of DNA replication. The other focus of our research is to identify specific *cis*-acting sequences and *trans*-acting factors that regulate DNA replication and gene expression at the beginning of mouse development.

Eukaryotic DNA replication

Kong, Saha, Vassilev, Zhang, DePamphilis; in collaboration with Coleman

During the past year, we have sought to determine whether ORC recognizes specific, genetically required sequences in complex eukaryotic replication origins and whether its activity is regulated as a function of the cell division cycle. The current paradigm for eukaryotic replication origins is the budding yeast *Saccharomyces cerevisiae,* whose replication origins consist of approximately 0.1 kb regions that contain a required consensus sequence to which ORC binds and an easily unwound DNA region where DNA synthesis begins. In contrast, replication origins in mammalian cells are much larger and lack a required consensus sequence. Moreover, even though replication origins do contain required sequences and ORC is bound to specific sites *in vivo*, site-specific ORC binding has not been observed *in vitro*. To understand the function of large complex eukaryotic replication origins, we used the fission yeast *Schizosaccharomyces pombe*, which, like mammalian cells, contains large, AT-rich replication origins that lack a recognizable consensus sequence but nevertheless contain sequences required for replication. Others had shown that the N-terminal half of SpOrc4 contains nine AT-hook motifs that strongly bind to AT-rich DNA sequences. We found that, both *in vitro* and *in vivo*, SpORC binds to specific sites within *S. pombe* replication origins that are genetically required for origin activity and that site selection is determined solely by the SpOrc4 subunit. These sites consisted of asymmetric A:T-rich sequences (clusters of A or T residues on one strand) but were devoid of either alternating A and T residues or GC-rich sequences. DNA-binding specificity was independent of both ATP and the remaining five SpORC subunits. We further found that SpOrc4p binds specifically to only two of the four required sequences in the *S. pombe* replication origin ARS3001. A pre-RC is assembled adjacent to the strongest ORC binding site, with the latter becoming the site at which bidirectional DNA replication begins. The second, and weaker, SpOrc4 DNA-binding site may facilitate pre-RC assembly, and the fourth appears to bind to a non-ORC protein that is required for origin activation. Finally, we were able to show that, during its replication in frog egg extract, *Xenopus laevis* ORC preferentially binds to the same AT-rich sites in sperm chromatin as are targeted by SpOrc4. The results suggest that fission yeast replication origins provide a more appropriate paradigm for the complex replication origins in multicellular animals than those of *S. cerevisiae*. These and other results from our and other laboratories imply that site-specific DNA replication in multicellular organisms results from both genetic and epigenetic parameters. ORC preferentially binds to asymmetric A:T-rich sequences, but, during animal development, some of the sites are selectively activated while others are suppressed.

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Regulation of ORC activity

Li, Sun, DePamphilis; in collaboration with Coleman

Binding of ORC to DNA (chromatin) is the first step in assembly of a prereplication complex. In contrast to yeast, in which all six ORC subunits are stably bound to chromatin throughout the cell cycle, mammalian Orc1 is selectively released from chromatin during S phase. Moreover, the Orc1 released during S phase is rapidly ubiquitinated and in some cases degraded. Other ORC subunits remain stably bound to chromatin and are not substrates for ubiquitination. During the M to G1 transition, Orc1 rebinds tightly to hamster ORC/chromatin sites to allow assembly of prereplication complexes, sites that are located at specific genomic loci referred to as "origins of bidirectional replication." The role of ubiquitination is to sequester Orc1 during S phase and thus prevent reinitiation at replication origins during a single cell division cycle, providing a mechanism for reprogramming replication origins during animal development or as a result of DNA damage in which the Orc1 subunit could be degraded.

In searching for the trigger that releases Orc1 from mammalian chromatin, we discovered two parameters that regulate the affinity of ORC for DNA in a cell cycle–dependent manner. First, the affinity of *Xenopus* ORC for DNA depends on chromatin structure. With sperm chromatin, XlORC remains bound when pre–RCs are assembled, but its affinity becomes salt-sensitive, and it is released only during mitosis. With somatic cell chromatin, XlORC is released as soon as the preRC is assembled. These results clearly demonstrated the existence of an "ORC cycle" that can regulate initiation of DNA replication during the cell cycle.

In mammalian cells, ORC activity is regulated during cell division by selectively releasing the Orc1 subunit during S phase and then preventing it from rebinding stably to chromatin until cyclin B is degraded, mitosis is completed, and a nuclear membrane is assembled. We have found that Orc1 is selectively and stably bound to a protein kinase during the G2/M-phase of proliferating hamster cells. We identified this Orc1-associated protein kinase as Cdk1(Cdc2)/cyclin A by its cell cycle specificity, ATP-binding, size, antibody recognition, immunoprecipitation with either anti–Orc1 or anti–Cdk1 antibodies, and sensitivity to inhibitors. It phosphorylated Orc1 *in vitro*, thereby accounting for the hyperphosphorylated Orc1 in M phase cells that was dephosphorylated during the M to G1 transition. Orc1 in M phase cells did not associate with either Orc2 or chromatin, but inhibition of Cdk activity resulted in rapid binding of Orc1 to chromatin. Thus, in mammals, premature rebinding of Orc1 to ORC/chromatin sites during mitosis appears to be prevented by the activity of Cdk1/cyclin A.

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Gene expression at the beginning of mammalian development

Vassilev, Kaneko, Zhang, Li, DePamphilis

To investigate gene expression at the beginning of mouse development, we have previously microinjected plasmid DNA into the nuclei of oocytes, fertilized eggs, and two-cell embryos and then measured the ability of these cells to either replicate the DNA or express a reporter gene. Our work led to the discovery of a novel transcription factor, mTEAD-2, that is expressed at the onset of zygotic gene expression (ZGE), at which point it is capable of strongly stimulating transcription from promoters or enhancers that contain its sequence-specific binding site. mTEAD-2 is the only member of the TEAD family of transcription factors that is expressed in mouse embryos during the first seven days of development. We identified the long sought-after coactivator of the TEAD family of transcription factors as YAP65; YAP65 interacts specifically with the C-terminus of all four TEAD proteins. Both this interaction and sequence-specific DNA binding by TEAD were required for transcriptional activation in mouse cells. Intriguingly, while TEAD is concentrated in the nucleus, YAP65 accumulates in the cytoplasm as a complex with the cytoplasmic localization protein 14-3-3. Given that TEAD-dependent transcription is limited by YAP65 and that YAP65 binds to Src/Yes protein tyrosine kinases, we propose that YAP65 regulates TEAD-dependent transcription in response to mitogenic signals. Our current goals are to identify the factors that regulate mTEAD-2 gene expression, to elucidate mechanisms by which mTEAD-2 regulates gene expression, and to identify the role of mTEAD-2 in mammalian development.

Investigation of the regulatory region of *mTEAD-2* led to the surprising discovery of another gene only 3.8 kb upstream of *mTEAD-2*. The new gene is a single copy gene called *Soggy* (*mSgy*) that is transcribed in the direction opposite to *mTEAD-2*, thus placing the regulatory elements of the two genes in proximity. *Soggy* (*Sgy*) and *Tead2*, two closely linked genes with CpG islands, are coordinately expressed in mouse preimplantation embryos and embryonic stem (ES) cells but are differentially expressed in differentiated cells. Analysis of established cell lines revealed that *Sgy* gene expression could be fully repressed by methylation of the *Sgy* promoter and that DNA methylation acted synergistically with chromatin deacetylation. Differential gene expression correlated with differential DNA methylation, resulting in sharp transitions from methylated to unmethylated DNA at the open promoter in both normal cells and tissues as well as in established cell lines. However, neither promoter was methylated in normal cells and tissues even when its transcripts were undetectable. Moreover, the *Sgy* promoter remained unmethylated as *Sgy* expression was repressed during ES cell differentiation. Therefore, DNA methylation was not the primary determinant of *Sgy/Tead2* expression. Nevertheless, *Sgy* expression was consistently restricted to basal levels whenever downstream regulatory sequences were methylated, suggesting that DNA methylation restricts but does not regulate differential gene expression during mouse development.

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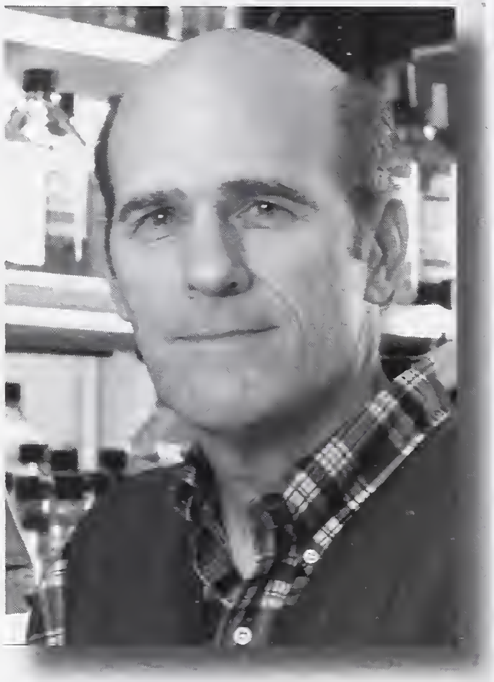
For more information and for illustrative figures, visit <*http://depamphilislab.nichd.nih.gov/*>

MAPPING HIGHER ORDER CHROMATIN STRUCTURES



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Our research focuses on the role of higher order chromatin structure in regulating gene expression. A topic of special interest is epigenetic memory, both how it is established and what determinants influence its stability. Our central hypothesis holds that errors in epigenetic memory contribute to developmental defects, cancer, and age-related diseases. Techniques under development assess the type and extent of chromatin remodeling that accompanies developmental programs, terminal differentiation, or senescence. In associated work, we perform structure-function studies to understand the roles played by specific chromatin regulatory gene products in establishing or maintaining epigenetic memory.

Genome sampling, bioinformatics, and high-throughput approaches to map higher order chromatin domains

Russanova, Howard

Several examples in the literature provide direct or indirect evidence for chromatin remodeling in conjunction with differentiation, cancer, or aging. Most such examples derive from serendipitous observations; thus, we have only a limited understanding of the extent and types of chromatin changes. Differential display is well established as a systematic means to screen for alterations in mRNA levels. We have combined chromatin immunoprecipitation (ChIP) with a modified form of differential display in order to screen for regions of unusual chromatin structure or domains of remodeling. In most genome sampling experiments to date, chromatin fragments in the ChIP step were fractionated according to histone acetylation level. Elevated levels of acetylation are typically associated with active gene transcription, whereas underacetylation correlates with repression or silencing. The human genome is organized into alternating gene-rich and gene-poor regions. An important validation of the ChIP genome sampling approach is provided by the observation that extremely underacetylated loci identified in the screens uniformly reside within gene-poor regions. By contrast, highly acetylated loci are found to lie predominantly within gene-rich regions. These differences are highly significant and would not be observed if chromatin fragment immunoprecipitation yielded loci at random with respect to higher order chromatin domains.

The HL-60 promyelocytic cell line has served as a useful model system in which to search for domains of chromatin remodeling. HL-60 cells differentiate into macrophage-like cells in response to treatment with phorbol esters. A screen of several thousand revealed several loci in which terminal differentiation along the macrophage lineage is accompanied by elevated histone acetylation. One such locus, located on chromosome 2 at a position 40 kb upstream from the EML4 gene locus, exhibits on average a six-fold increase in acetylation.

Searches for chromatin remodeling in the contexts of development and aging will require considerably improved approaches for mapping chromatin domains. To this end, we have developed an integrated system for high-throughput primer design, PCR product analysis, and graphic display of results. Novel regions of chromatin structure are likely initiated by combinations of sequence-specific DNA binding factors. To seed the mapping of such regions, we have developed a set of custom search algorithms that allow comparisons of mouse and human genomes for the presence of conserved binding sites. In excess of 100,000 sites per genome can be analyzed, followed by more than 10 billion blast comparisons, to yield potential binding sites of interest. The sites can be prioritized according to proximity to CpG islands, genes, and so forth. The *in silico* predictions can then be rapidly tested by chromatin immunoprecipitation experiments coupled with the high-throughput approaches noted above.

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Histone H3 methylation marks the murine H19-Igf2–imprinted region

Tchernov; in collaboration with Pfeifer, Stewart

The murine H19-Igf2–imprinted region is characterized by maternal expression of the H19 locus versus paternal expression of the Igf2 gene. A differentially methylated region (DMR) is located upstream from the H19 locus. In maternal cells, the DMR is unmethylated, binds to the transcription factor CTCF, and functions as an insulator to prevent activation of the Igf2 promoter by enhancers located downstream from H19. Conversely, in paternal cells, the DMR is methylated and, with respect to transcriptional and insulator activities, repressed. Histone H3 methylation at lysine position 9 (K9) appears to precede DNA methylation as a mark for transcriptional silencing in a number of systems. Experiments undertaken with collaborators revealed a high level of histone H3-K9 methylation near the H19-associated DMR and promoter region in paternal but not maternal cells. By contrast, H3-K9 methylation was detected at control regions for the Igf2 locus in paternal cells only. Of note, bisulfite mapping of DNA CpG-methylation sites confirmed that H3-K9 and DNA methylation occur with *opposite* parental bias upstream from the Igf2 gene. Moreover, the differential H3-K9 methylation defined an upstream region distinct from that marked by DNA methylation. This work reveals considerably greater complexity in the relative histone and DNA methylation patterns than previously appreciated.

Histone deacetylase and acetylase complexes

Bae; in collaboration with Ozato

As noted above, histone deacetylation is typically associated with transcriptional silencing. Numerous transcriptional repressors recruit RPD3-family deacetylases. Previous work by this group and others revealed that members of this family, including HDAC1, HDAC2, and HDAC3, function within multiprotein complexes to mediate repression. Complexes including the transcription factor co-REST together with HDAC1 or HDAC2, first delineated by this group, have emerged as highly important in nervous system development. The NAD-dependent deacetylase SIR2 is essential for silencing in budding yeast, and SIR2-like proteins comprise a highly conserved family ranging from yeast to mammals. Much remains to be learned as to whether Sirtuins, the mammalian SIR2 homologs, function in transcriptional regulation. Our experiments reveal that Sirtuin2 interacts with the homeodomain protein Hox-a10. We demonstrated interaction in the yeast two-hybrid system with *in vitro*–translated products and by coimmunoprecipitation from mammalian cell extracts. Overexpression of Sirtuin2 counteracts transcriptional activation by Hox-a10. The results establish a link between mammalian SIR2 homologs and transcriptional control; given that Hox-a10 plays a role in genito-urinary tract formation, it is possible that Sirtuin2 might modulate the establishment or maintenance of repressive chromatin domains during development.

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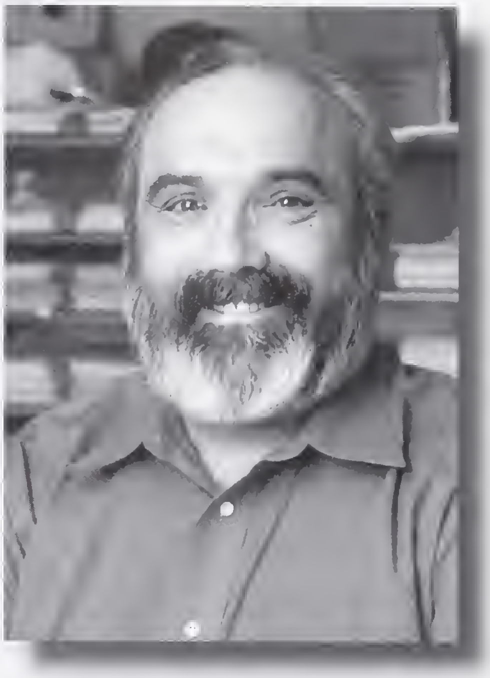
MECHANISMS OF EUKARYOTIC RNA EXPRESSION AND FUNCTIONS OF THE HUMAN LA ANTIGEN



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Using transcription by RNA-polymerase III (Pol III) as a model system of gene expression, we study mechanisms of eukaryotic RNA biogenesis and functions of the human La antigen. The Pol III transcripts studied most intensely are the transfer RNAs. The precursors of tRNAs are synthesized as nascent transcripts that undergo a "maturation" process involving a series of coordinated enzymatic and intracellular trafficking activities that ultimately deliver a functional tRNA to the site of protein synthesis in the cytoplasm, specifically, the ribosomes. We focus on transcription initiation and termination as well as on RNA processing and intranuclear trafficking, some of the steps in RNA production, and how the sequential steps are coordinated. We have been especially interested in the human La antigen, a protein that is a target of autoantibodies in patients suffering from certain autoimmune disorders (e.g., systemic lupus erythematosus). The human La antigen is a nuclear-cytoplasmic shuttling phosphoprotein that also binds to the 5′ untranslated regions of certain mRNAs and appears to regulate and coordinate critical steps in RNA expression. We use modern genetics, molecular biology, and biochemical approaches, relying heavily on analytic biochemistry as well as on tissue culture, yeast systems, and transgenic and gene-altered mice.

Functions of the human La antigen in RNA expression

Maraia, Intine, Schwartz, Treanor,[a] *Vech*[b]

Human La antigen is a nuclear phosphoprotein that so far has been found in all cells of all of eukaryotes. As alluded to above, La protein is a target "antigen" of antibodies (Ab) in patients suffering from autoimmune disorders such as systemic lupus erythematosous (SLE, lupus), neonatal lupus, and Sjögren's syndrome. Evidence from our and other laboratories indicates that La is a component of a Pol III holoenzyme that remains associated with newly synthesized transcripts in order to direct their maturation. As such, La is considered a regulatory chaperone for nascent RNAs, as it can control their nuclear residence and accessibility to the processing enzymes. La carries out its regulatory function, in part, by sequence-specific binding to a UUU-OH 3′ terminal motif that is common to all transcripts synthesized by Pol III and that results from transcription termination by RNA Pol III.

We mapped the major phosphorylation site of La to serine 366 (S366) and showed that the phosphorylation interferes with La's ability both to interact with the initiating pppG of the nascent transcript and to activate transcription initiation by Pol III, suggesting that such activities are mechanistically related. The implication is that an "La cycle" of transcription and posttranscriptional regulation is mediated in part by the C-terminal domain (CTD) of La. Others have recently shown that La is dephosphorylated at S366 early during apoptosis. We have developed two sets of monospecific antibodies that differ in their ability to recognize phosphoS366 (pLa) or nonphospohoS366 (npLa). The antibodies demonstrate that npLa and pLa exhibit different subnuclear localizations (nucleoplasm versus nucleoli) and that they are differentially associated with certain RNAs *in vivo*.

Our data indicate several trafficking signals in La that control nuclear, nucleolar, and cytoplasmic localization. Using a tRNA suppressor reporter system, we have identified an intranuclear trafficking defect that is associated with detrimental disordering of the tRNA processing activities. In this case, La recognizes and binds to its substrate pre-tRNAs normally but is deficient for proper routing in the nucleus, which causes the accumulation of a dead-end tRNA processing intermediate that is nonfunctional.

In revealing that La trafficks through the nucleus and is exported to the cytoplasm, our studies have uncovered an unexpected nuclear export signal (NES) in La. The finding of an efficient, carrier-mediated NES system for La proved surprising because the established functions of La in Pol III transcript biogenesis are entirely intranuclear. Further characterization revealed that the nuclear export pathway used for La as well as other features of its NES function are characteristic of some RNA binding proteins that carry certain mRNAs to the cytoplasm. Accordingly, we have begun to extend our studies to examine the involvement of La in the expression of certain target mRNAs, especially those whose expression is critical to growth and development.

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Transcription termination by RNA polymerase III

Maraia, Huang, Mozlin[c]

As indicated above, La protein binds to the 3′ terminal motif of nascent transcripts UUU-OH, which results from termination by RNA Pol III. We have developed a Pol III transcription system in the fission yeast *S. pombe*. A pol III–dependent gene encodes an opal suppressor tRNA that suppresses a nonsense codon in the mRNA encoding a purine-synthetic enzyme (*Ade6-704*), whose activity can be monitored by an *in vivo* colorimetric plate assay. We demonstrated that the expression of the gene in *S. pombe* is dependent on accurate and efficient termination by pol III and established that the minimal number of dT residues required for efficient termination is five. We have uncovered an intriguing correlation between the sensitivity of RNA Pol III to both the toxin alpha-amanitin and the Pol III termination signal and, using our *in vivo* reporter system, have begun a structure-function analysis of the largest subunit of Pol III. We have shown that our tRNA gene requires the La protein for efficient expression *in vivo*. Many important questions remain, such as the mechanistic link between La and pol III termination; whether the lack of reporter gene–derived transcripts in the La-minus strain are attributable to a defect in transcription rate, nascent RNA processing, or both; and whether other factors contribute to the La-dependent activation of this tRNA gene.

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Transcription initiation by RNA polymerase III

Maraia, Huang, McGillicuddy[d]

Although a longstanding interest of this section has been transcriptional termination, our recent discoveries have led us to examine mechanisms of Pol III initiation. Specifically, ongoing studies focus on the two central transcription factors (TF): TATA-binding protein (TBP) and the TFIIB-related factor Brf; both function at the core promoter. Homologs of these factors exist in all eukaryotes and archaea and are central in controlling transcription initiation. The core promoter is itself of central importance in transcription because, in addition to directing transcription initiation, core promoters integrate complex input from distal regulatory elements. Genomewide analysis has revealed that, in contrast to the core promoters of human and *S. cerevisiae* tRNA genes, which have long been known to be TATA-less, the core promoters of tRNA and 5S rRNA genes in *S. pombe* contain TATA elements. We used tRNA-dependent suppression and other *in vivo* assays as well as *in vitro* transcription to demonstrate an obligatory requirement for upstream TATA elements for tRNA and 5S rRNA expression in *S. pombe*. We found similar results for large rRNA synthesis, as mutation of the TATA element in the Pol I promoter also abolished rRNA expression in fission yeast, demonstrating TATA-unified transcription systems in eukaryotes and providing insight into the residual need for TBP by all three Pols in other eukaryotes, despite a lack of TATA elements in their promoters. We are using the yeast system to further our understanding of the basic mechanisms of transcription initiation.

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DEVELOPMENTAL GENE REGULATION OF THE IMMUNE SYSTEM

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We are interested in transcription factors that regulate the development of the immune system. We previously isolated IRF-8/ICSBP, a DNA-specific transcription factor expressed in hematopoietic cells, and showed that it regulates the development of bone marrow progenitor cells to generate macrophages and granulocytes and that it is critical for the development of dendritic cells (DCs) and the production of the type I interferons and IL-12, cytokines that are required for host defense. Using DNA microarray analysis, we have so far identified about 50 genes that are found either to increase or decrease following IRF-8 expression, some of which are important for macrophage/DC function. Studies of target promoters and the chromatin status of ICSBP-null cells suggest that IRF-8 regulates gene expression not only by controlling target promoters but also by regulating the activity of chromatin. We also study the role of chromatin from another perspective: the bromodomain protein Brd4. We showed that it associates with mitotic chromosomes, consistent with a strong affinity for chromatin. Using a proteomic approach, we have isolated a number of proteins that interact with Brd4, such as SPA-1, an activator of the small GTPase Rap, whose function is linked to Ras, a major regulator of mitogenic signaling pathways.

Interferon gamma triggers the interaction between ICSBP (IRF-8) and TEL, recruiting the histone deacetylase HDAC3 to the interferon-responsive element

Tamura, Kanno; in collaboration with Kuwata

ICSBP (IRF-8) is a transcription factor of the IRF family expressed only in the immune system. It is induced in macrophages by interferon gamma (IFNgamma) and critically contributes to macrophage functions. By interacting with an Ets family protein PU.1, ICSBP binds to the IRF/Ets composite element and stimulates transcription. ICSBP binds to another DNA element, the IFN-stimulated response element (ISRE), a common target of the IRF family. Despite much progress in the field, limited knowledge is available as to how ICSBP and other IRF proteins regulate ISRE-dependent transcription in IFNgamma-activated macrophages. Using mass-spectrometry analysis of ISRE-bound proteins in macrophages, we identified TEL, another Ets member, as a factor recruited to the element in an IFNgamma-dependent manner. *In vitro* analysis with recombinant proteins indicated that the recruitment is attributable to a direct interaction between ICSBP and TEL, which is enhanced by the presence of ISRE. Significantly, the interaction with TEL in turn resulted in the recruitment of the histone deacetylase HDAC3 to the ISRE, causing increased repression in IFNgamma-mediated reporter activity through the ISRE. Together, by associating with two different

Ets family proteins, ICSBP exerts a dual function in IFNgamma-dependent gene regulation in an immune system–specific manner.

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Role of ICSBP in the development of interferon-producing plasmacytoid dendritic cells

Tsujimura, Tamura, Kong, Tailor

DCs, a newly identified cell type important for establishing innate and adaptive immunity, develop from bone marrow (BM) progenitor cells and mature in response to external signals to elicit functions important for innate and adaptive immunity. We found that ICSBP$^{-/-}$ mice, in addition to their inability to produce type I interferons and IL-12, lack subsets of DCs (pDCs and CD8alpha^{+} DCs). To investigate the role of ICSBP in DC development, we employed an Flt3 ligand–based *in vitro* culture system. DCs developed *in vitro* from *ICSBP*$^{-/-}$ BM cells were defective in the expression of MHC class II and costimulatory molecules, indicating that differentiation of *ICSBP*$^{-/-}$ progenitors toward immature DCs is impaired. Moreover, -/- DCs displayed a generalized maturation failure and did not express type I interferon and IL-12 p40 mRNA in response to various maturation signals, consistent with the feature observed with *ICSBP*$^{-/-}$ DCs *in vivo*. Further, -/- DCs failed to increase expression of MHC class II and costimulatory molecules upon maturation signals. We showed that retroviral introduction of ICSBP restores the ability of -/- progenitors to develop plasmacytoid DCs that undergo full maturation upon activation signals. Transduction of the wild-type ICSBP, but not transcriptionally inactive mutants, increased expression of MHC class II and costimulatory molecules on immature cells. More important, ICSBP-transduced cells produced interferons and IL-12 proteins upon activation, along with increased expression of costimulatory molecules. Thus, introduction of ICSBP rescued not only the development of macrophages and DCs but also the expression of both cytokine genes. Our study identifies ICSBP as a factor critical for both early differentiation and final maturation of DCs.

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Bromodomain protein Brd4 binding to and modulation of the activity and subcellular localization of the GTPase-activating SPA-1

Farina, Dey

Brd4, a novel bromodomain-containing protein that binds to chromatin, regulates cell growth and affects cell cycle progression at multiple stages. By mass-spectrometry analysis, we identified the

GTPase-activating protein (GAP) SPA-1 as a factor that interacts with Brd4 in human cells. Brd4 and SPA-1 bound to each other *in vitro* through specific domains. SPA-1 regulates mitogenic signaling pathways by activating Rap1 and Rap2. Consistent with the functional importance of the interaction, Brd4 enhanced Rap GAP activity of SPA-1 *in vivo* and *in vitro*. SPA-1 localizes to the cytoplasm and, to a lesser degree, in the nucleus while Brd4 resides in the nucleus. We show that ectopic expression of Brd4 redirects subcellular localization of SPA-1 and vice versa, indicating that Brd4 and SPA-1 mutually influence their localization in the cell. Our work reveals a novel link between Brd4 and GTPase-dependent mitogenic signaling pathways. Functional analysis of Brd4-SPA-1 interaction indicates that it is part of growth-regulatory activity of Brd4 in G1/S transition. We are pursuing further elucidation of the role of this factor by conditional knock-out and antisense/RNAi approaches.

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Double bromodomain protein Brd4 binding to acetylated chromatin during interphase and mitosis

Dey, Ozato; in collaboration with Misteli

Previous studies on RXR led to the identification and characterization of Brd4. Recent FRAP and FLIP analyses combined with biochemical studies showed that Brd4 interacts with acetylated chromatin through the bromodomains in living cells as a consequence of its high affinity for acetylated core histones H4 and H3, leading to the proposal that the domain is involved in deciphering the histone code. However, little *in vivo* evidence supports the binding of bromodomains to acetylated chromatin in the native environment. Brd4 is a member of the BET family that carries two bromodomains. It associates with mitotic chromosomes, a feature characteristic of the family (Figure 18.1). We studied the interaction of Brd4 with chromatin in living cells by photobleaching. Brd4 was mobile and interacted with chromatin with a rapid "on and off" mode of binding. The interaction required both bromodomains. Indicating a preferential interaction with acetylated chromatin, Brd4 became less mobile upon increased chromatin acetylation caused by a histone deacetylase inhibitor. Providing biochemical supporting evidence, the salt solubility of Brd4 was markedly reduced upon increased histone acetylation. This change also required both bromodomains. In peptide binding assays, Brd4 bound avidly to di- and tetraacetylated histone H4 and diacetylated H3 but weakly or not at all to mono- and unacetylated H3 and H4. By contrast, it did not bind to unacetylated H4 or H3. Further, Brd4 colocalized with acetylated H4 and H3 in noncentromeric regions of mitotic chromosomes. This colocalization also required both bromodomains. Our observations indicate that Brd4 specifically recognizes acetylated histone codes, with such recognition passed on to the chromatin of newly divided cells.

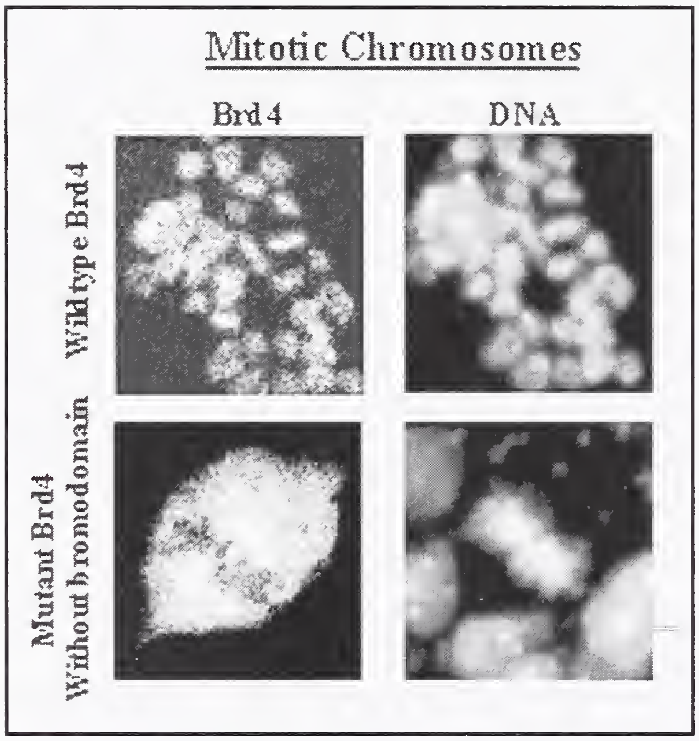

FIGURE 18.1 Double bromodomain protein Brd4

Brd4 associates with mitotic chromosomes. P19 cells were transfected with wild-type GFP-Brd4 or the Brd4 mutant lacking bromodomains, and mitotic cells were visualized by confocal microscopy. Condensed chromosomes were stained with Hoeschst.

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BIOPHYSICS OF LARGE MEMBRANE CHANNELS

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We study channel-facilitated transport of metabolites and other large solutes across cell and organelle membranes. Membrane proteins respond to their immediate environment such as, for example, surrounding lipids and local acidity. Therefore, to study channels under precisely controlled conditions, we reconstitute channel-forming proteins into planar lipid bilayers. These proteins include VDAC (voltage-dependent anionic channel) from the outer membrane of mitochondria, OmpF (general bacterial porin), LamB (sugar-specific bacterial porin), alpha-hemolysin (channel formed in the target cell membranes by a toxin from *Staphylococcus aureus*), alamethicin (oligomeric peptide channel), and syringomycin E (oligomeric lipopeptide channel). The channels have aqueous pores of 1 nm or larger in diameter. Emerging evidence demonstrates that, rather than functioning merely as "general diffusion pores," most of these large channels are structures with a high specialization for the solutes they allow to cross membranes. Our goal is to unveil the main physical principles of channel selectivity to solutes other than small ions. Such knowledge will advance our understanding of the molecular mechanisms responsible for metabolite flux regulation under normal conditions and in pathology.

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Theory of channel-facilitated solute transport

Bezrukov; in collaboration with Berezhkovskii, Pustovoit

Recent experimental and theoretical work demonstrates that solute translocation can be facilitated by attractive interactions between the channel and the penetrating particle. Although the constructive role of attractive interactions between permeating particles and the channel has been appreciated for many years, a comprehensive theory capable of offering a clear understanding and reliable quantitative description of the channel-facilitated passive metabolite transport is still to be developed. As an important step toward such a theory, we considered, this year, the average lifetimes in the channel for those particles that traverse the channel and those that return as well as the total average lifetime of the particle in the channel. Exact expressions for the average lifetimes have been derived in the framework of a one-dimensional diffusion model. The validity of our one-dimensional approach was verified by good agreement of the theoretical predictions with the average lifetimes found in three-dimensional Brownian dynamics simulations.

To illustrate some qualitative features of the average lifetimes predicted by the general theory, we studied a special case in which a symmetric square-well potential occupies some part of the channel length. We find that both the total average lifetime in the channel and the average return time are monotonically increasing functions of the well depth and length. Such behavior agrees with general intuitive ideas. In contrast, the dependence of the average translocation time on the well length is somewhat counterintuitive. The time increases with the length when the length is small, reaches a maximum when the well occupies half of the channel, and then starts to decrease. Concerning the dependence on the well depth, the average translocation time monotonically increases with the depth. The deeper the well, the more pronounced is the turnover behavior of the translocation time.

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Water-soluble polymers as molecular probes for ion channels

Stojilkovic, Bezrukov; in collaboration with Berezhkovskii, Zitserman

Water-soluble polymers have proven to be valuable probes of ion channels. By analyzing the channel conductance in the presence of polyethylene glycol (PEG), it is possible to investigate channel geometry. Understanding the mechanism by which PEGs reduce electrolyte conductivity is important for the interpretation of our experiments. This year, we completed a study of polymer-induced variation of the electrolyte conductivity in the bulk solutions. We measured the conductivity of potassium chloride solutions containing PEGs of different molecular mass in a wide range of the polymer concentrations up to 33 weight percent for PEG 300, 600, 2000, 4600, and 10000. We used the data to find the dependence of microviscosity, which characterizes the decrease of the ion mobility compared with that in the polymer-free solution, on the polymer volume fraction. We found that the dependence is well approximated by a simple exponential relation that expresses the microviscosity of the polymer-containing solution through the polymer volume fraction. Parameters of the relation only weakly depend on the polymer molecular mass. Through these parameters, we can derive practical formulae for the PEG effect on electrolyte solution conductivity, formulae that will be helpful in future work with ion channels.

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Residue ionization and ion transport through large channels

Nestorovich, Rostovtseva, Bezrukov

New methods developed for investigations of metabolite and other large-molecule transport at the single-channel level require detailed knowledge of the stability and noise characteristics of the open single channels. Appreciation of noise generation mechanisms in nanoscale objects is also important for the successful development of single-molecular sensors based on natural ion channels and solid-state micro- and nanostructures. This year, to address these questions, we studied conductance, selectivity, step-wise current transients, and open-channel noise of single trimeric channels of the general bacterial porin OmpF, over a wide range of proton concentrations. We found that OmpF channels reconstituted in planar bilayer membranes can endure extreme pH conditions for hours. Exposed to highly acidic or basic environment of pH 1 or pH 12, they can be returned to their completely functional state when solution pH is brought back to neutral. Such robustness makes it possible to study channel transport properties over a broad range of solution pH. From pH 1 to pH 12, channel transport displays three distinct characteristics. First, in acidic solutions, channel conductance is a strong function of pH; it increases about three-fold as the proton concentration decreases from pH 1 to pH 5. This rise in conductance is accompanied by a sharp increase in cation transport number and by pronounced open-channel low-frequency current noise with a peak around pH 2.5. Random stepwise transients with amplitudes about one-fifth of the monomer conductance are major contributors to the noise. Over the middle range (pH 5 to pH 9), channel conductance and selectivity stay virtually constant; open-channel noise is at its minimum. Over the basic range (pH 9 to pH 12), channel conductance and cation selectivity start to grow again with an onset of a higher frequency open-channel noise. We attribute these effects to the reversible protonation of channel residues whose pH-dependent charge influences transport by direct interactions with the ions passing through the channel.

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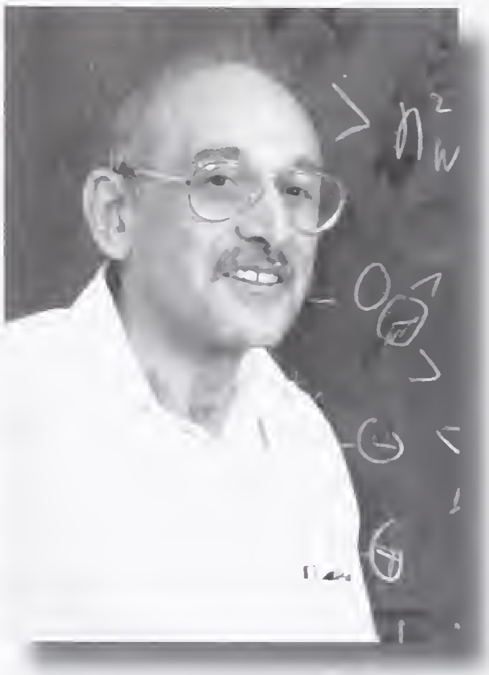
PHYSICAL FORCES ORGANIZING BIOMOLECULES



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Despite the remarkable advances in molecular biology, the tools for describing how biomolecules do their work remain primitive. We therefore measure, characterize, and codify the forces that govern the organization of a wide variety of biological molecules. Our undertaking is strengthened by its strong connection with physical theory. Our long-term goal is to build a practical physics of biological material. To that end, we undertake a series of measurements and analyses of the different types of forces as revealed *in vivo, in vitro,* and in computation. In particular, we are working with DNA/lipid assemblies for gene therapy; DNA assemblies such as are seen in viral capsids and *in vitro*; polypeptides and polysaccharides in suspension; and lipid/water liquid crystals. In all these systems, we simultaneously observe the structure of packing and measure intermolecular forces or interaction energies.

Ions, lipids, and membrane-protein interactions

Petrache, Podgornik; in collaboration with Dubois, Gawrisch, Nagle, Zemb

Given that ions vary widely in their effects on biological materials, ion "specificity" beyond simple charge properties is a major issue in biology. One overlooked property of ions is polarizability, the ability of the charge to shift or fluctuate, a property seen in charge fluctuation forces. Another surprising feature of ions is their tendency to stick to charged bilayers to an extent beyond what is expected from charge-charge attraction. This stickiness changes the way membranes interact; it also introduces strains that can alter the way proteins are accommodated and are able to change conformation as in the opening and closing of transmembrane ionic channels. Our comparisons between bilayers of phosphatidylserine (PS) and phosphatidylcholine (PC) lipids with the same chains and at the same temperature enable us to focus on the effects of these headgroups on bilayer properties.

Using X-ray diffraction and NMR spectroscopy, we found that, in the presence of sodium ions, negatively charged PS bilayers have lateral areas much smaller than the areas of corresponding neutral PC analogs. The shrinkage occurs despite the extra electrostatic repulsion expected for charged PS headgroups. The condensation of area suggests an extra attractive interaction, perhaps hydrogen bonding, between PS headgroups. We find that the charged bilayers repel as expected when interlamellar forces are measured by osmotic stress and X-ray diffraction. The questions arise as to why the same repulsion does not occur between charged groups on the same bilayer and why the charged lipids are stiffer against bending. The culprit is likely to be strong ion binding. Indeed, we found a few years ago that protons bind so strongly to these same lipids that, at 10^{-4}

Molar concentrations (pH 4), the membrane is wrenched from the lamellar form into an inverted hexagonal structure. Even more intriguing is the observation that, when these same lipids are used as a scaffold for protein channels, exposure to protons shifts channel gating.

We have thus become aware of the organizing power of small ions changing membrane protein behavior. Guided by the unexpectedly strong attraction between charged phospholipids and simple ions, we have begun to examine Li ions with PS bilayers, building on earlier work by others suggesting that Li acts like protons. We are now asking whether Li can stress lipids assembly and, consequently, whether low levels of Li modify channel gating. Structural studies have progressed during the past year, including work at CHESS (Cornell High Energy Synchrotron Source), where we obtain high-resolution data needed for measurement of fluctuations, forces, and stress in protein-hosting lipids.

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Van der Waals forces

Parsegian, Petrache, Podgornik; in collaboration with French, Nagle

It is not generally appreciated how much work is done among membranes and macromolecules by bonds that are weaker than covalent bonds, electrical-charge interactions, charge fluctuation, or van der Waals forces. The main force that coheres membranes and proteins and a source of the powerful surface tension at membrane interfaces, van der Waals forces are the dominant, perhaps sole, attraction that creates membrane multilayers or allows membranes to adhere to artificial surfaces. This past year, we have learned to formulate, measure, and modify these neglected interactions. The key has been to begin with the elements of physical theory and to relate the polarizability of materials to the fluctuations of charges within them. From this, we have been able to design experiments that show how macromolecular organization responds to deliberate changes in solution properties.

We have been able to see how adding even the simplest salts to water around lipid bilayers will change how these membranes assemble into multilayers. For example, we have seen how the attraction between membranes varies when salts of different ions (e.g., chloride versus bromide) are dissolved in the intervening water. Membrane multilayers will swell by 50 percent with bromide but not with chloride. We have reformulated van der Waals forces between membranes to show how they would respond to changes in solutions as a strategy to control membrane assembly. One unexpected byproduct has been a collaboration with engineers using our equations to design production procedures for thin-film resistors in computer chips. We expect the collaboration to work to our benefit by providing us with experimental data that can be used to compute van der Waals forces. Another unexpected result relates to what was known about the friction between an ion and the water through which it moves. Just as a charge-water interaction creates a drag, there is a charge-fluctuation source of drag on a moving particle.

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Cosolutes in molecular folding and association

Harries, Parsegian; in collaboration with Rau

Driven by the conditions set by smaller solutes, proteins "fold" and "unfold." Experimentally, these conditions are stated as intensive variables, i.e., pH and other chemical potentials, as though small solutes were infinite resources that come at an independently varied price. Computationally, the finite spaces of simulation allow only fixed small numbers of the solutes. By combining the analytic Gibbs adsorption isotherm with the computational Monte Carlo sampling of polymer configurations, we have been able to overcome an inherent limitation of computer simulation. The key is to compute analytically the free energy changes wrought by solutes on each particular configuration. Then, numerical computation is needed only to sample the set of configurations as efficiently as though no bathing solute were present. The result is a more accurate computation procedure 500 times faster than earlier simulations that had to count all possible positions of the cosolutes in the bathing solution.

To monitor how small, adherent molecules affect molecular association, we measured the changes of binding free energy versus change in water activity for the specific binding of cyclodextrin with an adamantane derivative. Using different salts and neutral agents, we found that the dependence of the binding constant on osmotic pressure suggests a release of 15 to 25 water molecules from the interacting surfaces upon association, depending on the type of solute used. The observed dependence of binding free energy and enthalpy with added solute indicates that these osmolytes are interacting primarily enthalpically with these surfaces.

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INTERMOLECULAR FORCES, RECOGNITION, AND DYNAMICS

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Our laboratory focuses on elucidating the coupling of forces, structure, and dynamics of biologically important macromolecules. One of the immediate challenges in structural biology is to understand the physics of interactions between molecules in aqueous solution. The ability to take advantage of the increasing number of protein and nucleic acid structures determined by X-ray crystallography and solution NMR will depend critically on knowledge of such interactions, as will our understanding of the strength and specificity of interactions among biologically important macromolecules that control cellular function and our ability to design rationally agents that can effectively compete with those specific interactions that are associated with disease. Our earlier results have shown that experimentally measured forces differ markedly from those predicted by current, conventionally accepted theories of intermolecular interactions. We have interpreted the observed forces as indicating a dominant contribution from water-structuring energetics. We use osmotic stress and X-ray scattering to measure directly forces between biological macromolecules in macroscopic condensed arrays. As a first step toward linking our observation of dominant hydration forces between surfaces in condensed arrays to the interaction of individual molecules in solution, we also measure changes in hydration accompanying specific recognition reactions of biologically important macromolecules in dilute solution.

Direct force measurements

Rau; in collaboration with Hansen, Parsegian, Podgornik, Yang

We previously showed that hydration interactions lead to a strong exclusion of salts and polar solutes from the nonpolar polymer hydroxypropyl cellulose. The biological consequences of the strength and ubiquity of these interactions are such that the thermodynamics of assembly and recognition reactions that occur within the crowded environment of a cell will differ noticeably from those in dilute solution. It also means that hydration or water-structuring interactions will likely affect the distribution of small solutes and salts around all macromolecules. In particular, the distribution of salts around charged macromolecules is likely to differ from predictions based on conventional electrostatic theories. To test these assumptions, we made extensive measurements of forces between DNA helices with varying NaBr and tetramethylammonium bromide (TMABr) concentrations. Changes in the number of ions in the DNA phase as the distance decreases between helices can be calculated through the fundamental Gibbs-Duhem relationship of basic thermodynamics. Our initial analysis suggests that, while the distribution of NaBr around DNA appears dominated by electrostatics, the distribution of TMABr salt appears determined by strong hydration repulsion.

This reasoning may account for our previously puzzling results for the interactions between highly charged, double-helical iota-carrageenan polysaccharides. Unlike DNA, this polymer has charges bound to a hydrophobic backbone. The repulsion of salt from the backbone due to hydration forces seems to overwhelm the electrostatic interactions at near distances. The ability to combine osmotic stress and fundamental thermodynamics to derive the distribution function of small solutes and salts around macromolecules in condensed arrays offers an unprecedented opportunity to formulate a physics for molecular interactions at close distances based on experimental observation.

We have also uncovered an unusual feature of spermidine-induced assembly of DNA. Spermidine, a trivalent cation, is an effective agent for condensing DNA into closely packed structures. At 2 mM spermidine and in the absence of salt, DNA helices assemble into hexagonally packed arrays with an interaxial spacing of 29.5 Å. With increasing concentration of spermidine, NaCl, or betaine glycine, we see evidence for a second phase with an interhelical spacing of about 33.5 Å. Indicative of a much shallower attractive energy minimum, the new peak is much broader than the reflection at 29.5 Å. The two structures coexist over a limited range of concentrations. A similar transition is not observed for cobalt hexammine–condensed DNA even up to very high trivalent salt concentrations. As the concentration of spermidine is increased even further, DNA dissolves into the solution as an abrupt transition. We have also measured the transition between states as a function of osmotic pressure and spermidine concentration. The stronger energy minimum at about 29.7 Å could reflect highly ordered, intercorrelated arrays of helices with spermidine bound at localized sites (for example, in grooves) to maximize attractive interactions. The transition to the weaker state could then reflect a movement of spermidine to other binding sites that are not as attractive or a loss of correlated binding between helices. Alternatively, with high salt or spermidine concentrations, a substantial mix of +2 and +3 spermidine species may be attributable to deprotonation or Cl^- binding. The weaker attractive minimum may represent a particular mix of di- and tri-ion binding. Before we can understand the interplay of salt and spermidine, we need to know more about the spermidine species that are present in solution under our conditions, in particular Cl^- binding.

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Water and DNA-protein recognition reactions

Sidorova, Rau

We have continued investigating the role of water in the sequence-specific recognition reaction of the restriction nuclease EcoRI. The exceptionally stringent specificity of the enzyme is a paradigm for recognition reactions. We measure the number of waters coupled to a binding reaction from the dependence of the binding free energy on water chemical potential or, equivalently, osmotic pressure. We have previously found that a nonspecific complex of the protein sequesters about 110 more waters than the specific one and that the water is likely at the protein-DNA interface. We have further shown that the dissociation rate of the specific complex is also highly sensitive to osmotic pressure, requiring the net binding of 120 to 150 waters.

During the past year, we have investigated more closely how the number of waters sequestered depends on DNA sequence. Sequences that differ by even a single base pair ("star" sequences) from the specific recognition sequence (GAATTC) of EcoRI bind only slightly better than completely nonspecific sequences. This abrupt decrease in binding energy with even a single base pair change is accompanied by an abrupt increase in the water sequestered by the EcoRI-DNA complex. At low stresses, the osmotic sensitivities of both the competitive equilibrium constants and the dissociation rates of complexes with two "star" sequences and with nonspecific DNA are practically indistinguishable. At low osmotic stresses, the complexes with noncognate sequences sequester a similar number of waters (110) as nonspecific complexes. At least some of this water, however, can be removed from these noncognate complexes by applying sufficiently high osmotic pressures. Given that equilibration times at high stresses are too long for convenient measurement of binding constants, we have used dissociation rates to determine loss of sequestered water. At higher pressures, the behavior of "star" sequence complexes is strikingly different from that of the nonspecific complex, with a clear correlation between the osmotic dependence of the dissociation rate and DNA sequence. The kinetic measurements show that the TAATTC "star" sequence complex (strongest "star" site for EcoRI) loses about 90 of its initially sequestered waters at osmotic pressure of 150 atmospheres. The osmotic work required to remove these waters is about 5 Kcal/mole. Over the same range of pressures, the complex with the weaker "star" site, CAATTC, loses only about 20 to 30 of its waters, with no apparent loss of water from the nonspecific complex. The amount of water we are able to remove from a noncognate complex correlates with the ability of the enzyme to cleave the sequence. In principle, any sequestered water can be removed by applying sufficiently high osmotic stress, but the work necessary to dehydrate complexes naturally depends on resulting DNA-protein contacts.

We are beginning to investigate the correlation between water release and DNA binding strength for another restriction endonuclease, Bam HI. The significant advantage of this system is that X-ray structures are available for both specific and nonspecific complexes of the BamHI with DNA. The nonspecific complex has lost all direct contacts with the bases in the major groove. Despite extensive contacts between the sugar-phosphate backbone and the protein, a large cavity is seen between the major groove of the DNA and the recognition protein surface. The volume of the cavity is equivalent to about 150 waters. Initial measurements indicate the nonspecific BamHI-DNA complex sequesters about 100 waters.

We have also started to investigate the correlation between water and binding strength of DNA complexes with the lambda Cro repressor. The protein is responsible for the induction of the lytic phase of lambda phage. The recognition stringency for this repressor is more typical of specific-sequence DNA-protein binding systems. Single base pair changes from the optimal recognition sequence reduce binding energies by only a few kT. We have constructed a series of DNA fragments with consensus and altered Cro recognition sequences whose binding constants span a range of about 1,000. The initial results for the osmotic pressure dependence of the dissociation rate for the various complexes reveal a surprising linear relationship between binding energy and sequestered water. For every factor of 10 decrease in the binding constant, the complex sequesters an extra 15 water molecules.

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THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS: ITS ENDOCRINE DISORDERS AND ITS INTERACTIONS WITH THE IMMUNE SYSTEM

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We investigate the molecular basis of genetic disorders of the hypothalamic-pituitary-adrenal (HPA) axis, such as pituitary and adrenal tumors, adrenal insufficiency, ACTH resistance, congenital adrenal hyperplasia (CAH), glucocorticoid resistance/hypersensitivity, and mineralocorticoid resistance; diseases of the hypothalamic-pituitary-gonadal (HPG) axis, such as hypothalamic hypogonadism; and metabolic and immune disorders related to the functions of the HPA and HPG axes and the autonomic system, such as premature adrenarche, eating disorders that include anorexia and bulimia nervosa and adolescent obesity, childhood asthma and rheumatoid arthritis, premenstrual tension syndrome, and postpartum and climacteric depression and autoimmunity. We also study the critical influences of early life stress on the later development of behavioral disorders (dysthymia, depression, dissociative and conduct disorders, substance abuse) and metabolic disorders (metabolic syndrome X). By identifying pathogenetic defects of the adrenocorticotropic hormone (ACTH) and glucocorticoid receptor genes and of genes of functionally related molecules, we have elucidated the molecular mechanisms of the diseases congenital isolated glucocorticoid deficiency and glucocorticoid resistance syndromes and of acquired states such as glucocorticoid resistance associated with respiratory distress syndrome or glucocorticoid hypersensitivity associated with the AIDS-related lipodystrophy and insulin resistance syndrome. We also study the physiologic and pathophysiologic interactions between the endocrine and immune systems.

A natural determinant of glucocorticoid sensitivity and its role in pathophysiology

Chrousos, Kino, de Martino, Charmandari, Souvatzoglou, Vottero,[d] *Alesci*

The glucocorticoid receptor gene, located in the long arm of chromosome 5, encodes two proteins produced by alternative splicing: the classic glucocorticoid receptor, designated GRalpha, and a nonligand-binding variant called GRbeta. We demonstrated that both isoforms are expressed in human tissues and that the nonligand-binding receptor is present at high concentrations in normal human tissues and exerts dominant negative effects on the classic receptor. Given that alteration in glucocorticoid receptor gene splicing produces different ratios of GRalpha to GRbeta, we hypothesized that the ratio of glucocorticoid receptor types regulates tissue sensitivity to glucocorticoids. A change in sensitivity might have major implications for human pathophysiology in that both glucocorticoid resistance and glucocorticoid hypersensitivity can produce disease states. The former causes hyperandrogenism and hypertension while the latter could be involved in metabolic disorders such as visceral obesity or syndrome X, affective disorders such as depression, and immune disorders characterized by suppression of the inflammatory/immune response and vulnerability to infectious diseases and/or tumors. We showed that glucocorticoid-resistant asthma type II is associated with a major shift in the GRalpha-to-GRbeta ratio toward GRbeta predominance, explaining the profound resistance of patients' cells to glucocorticoids. We observed a similar shift in cells from glucocorticoid-resistant New World monkeys. We demonstrated that normal human neutrophils, which are naturally glucocorticoid-resistant, also overexpress GRbeta. Recently, we showed how the GRbeta isoform causes glucocorticoid insensitivity. This isoform forms heterodimers with GRalpha, which then inhibit interaction with the nuclear receptor p160 transcription coactivators and, hence, the transcription of glucocorticoid receptor-responsive genes.

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Viral determinants of glucocorticoid hypersensitivity in patients with AIDS and AIDS-related lipodystrophy and insulin resistance syndrome

Chrousos, Kino, de Martino, Charmandari, Souvatzoglou, Vottero,[d] Alesci

Recently, we described a human example of a glucocorticoid hypersensitivity state: one of the accessory proteins of the HIV-1, called Vpr, acts as a coregulator of the glucocorticoid receptor, altering the sensitivity of human cells to glucocorticoids so that viral replication is favored. Such hypersensitivity explains several manifestations of AIDS, including innate and T helper 1 immunosupression, T helper 2 shift, myopathy and muscle atrophy, and visceral obesity–related insulin resistance with its metabolic manifestations and cardiovascular consequences. Vpr coactivates the glucocorticoid receptor by binding to it and to the cointegrator molecule p300/CBP and by stabilizing the transcription initiation complex. Recently, we identified a second HIV-1 accessory protein called Tat that also acts as a coactivator of the glucocorticoid receptor. This protein binds to nuclear receptor p160 coactivators and cooperates with Vpr in potentiating the activity of glucocorticoids on transcription of responsive genes. It appears that the HIV-1 LTR promoter uses p300/CBP and p160 coactivators for its own activation as well. The ability of HIV-1 proteins such as Vpr to influence the activity of nuclear receptor coactivators extends beyond the glucocorticoid signaling system. We recently identified the PPAR-gamma and the insulin signaling systems as targets of Vpr in the host cell. Indeed, Vpr causes resistance to PPAR-gamma and insulin by directly interfering with the actions of the former on transcription and by indirectly blocking insulin actions on the activity of the fork-head transcription factor, a major modulator of insulin actions.

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Comparison of familial and sporadic glucocorticoid resistance syndromes with inflammation-induced glucocorticoid resistance

Chrousos, Kino, de Martino, Charmandari, Souvatzoglou, Vottero,[d] Alesci; in collaboration with Meduri, Latronico

Our group recently analyzed patients with the familial/sporadic glucocorticoid resistance syndrome, which is caused by mutations of the glucocorticoid receptor gene. Two novel glucocorticoid receptor mutations, one replacing isoleucine (I) 559 with aspartic acid (N) and another in which isoleucine (I) 747 was replaced by methionine (M), both caused glucocorticoid resistance in the heterozygote state, acting in a transdominant negative fashion by forming function-deficient dimers with wild-type GRalpha. We examined the cause of the transdominant activity of these mutant receptors and found that GRI559N has a nuclear localization sequence defect that interferes with the translocation of wild-type GRalpha from the cytoplasm into the nucleus while GRI747M has a defective Activation Function–2 (AF-2) domain that prevents proper interaction of the wild-type receptor with the p160 nuclear receptor coactivators. Recently, we studied a newborn female infant with severe sexual ambiguity, a novel presentation of the glucocorticoid resistance syndrome. In this patient, the GRalpha gene bore a homozygous mutation that decreased its affinity for glucocorticoids and its ability to activate glucocorticoid-responsive genes. We are studying an American kindred in which the proposita has a heterozygote amino acid substitution in its C-terminal, disturbing AF-2–mediated interaction with p160 nuclear receptor coactivators.

We have further studied the mechanisms by which severe inflammation causes resistance of immune and immune-associated cells to glucocorticoids. We have determined that the critical mechanism is an inflammatory cytokine–induced signaling cascade in which the TNF alpha and Fas system (1) activate NF-kappaB by phosphorylation and nuclear translocation and (2) block the p160 glucocorticoid receptor p160 coactivators from interacting properly with the glucocorticoid receptor through activation of an intermediate cytoplasmic protein called FLASH.

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Pathophysiology and therapy of adrenal hyperfunction–congenital adrenal hyperplasia and other congenital endocrine disorders

Chrousos, Merke, Charmandari

The goal of our research is to understand the mechanisms involved in diseases caused by adrenal hyperandrogenism. Deborah Merke is principal investigator for a clinical trial that is testing a treatment for children with classic congenital adrenal hyperplasia (CAH). Preliminary results based on two years of therapy showed that children receiving the treatment had normalization of growth velocity and bone maturation. Thus, the regimen represents a promising treatment for children with classic 21-hydroxylase deficiency. Reproductive, metabolic, and other morbidities, including risk of tumor formation, particularly testicular tumors, are currently under investigation in the severe forms of the disease. Bilateral adrenalectomy performed on a severe case revealed massive lymphocytic infiltration and histologic signs of adrenal carcinoma. Recent clinical studies revealed adrenomedullary dysplasia and hypofunction in patients with CAH and that the protective function of the adrenal medulla may be absent in the most severe cases. Studies of hypoglycemia and exercise tolerance in patients are under way. It appears that the lack of a proper epinephrine response to exercise prevents an appropriate glucose response in CAH. Recently, children with CAH were found to be resistant to insulin. Preliminary results from studies of healthy children reveal sex and age differences in catecholamine secretion. New clinical initiatives include studies of the pathophysiology of premature adrenarche and the effects of androgens and other hormones on the brain. Psychological testing and head MRI studies are being conducted with children suffering from endocrine disorders, including CAH, Cushing's syndrome, and familial male precocious puberty. The studies will provide insight into hormonal influences on the brain during growth and development.

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Role of CRH in behavior and behavioral/developmental/sleep disorders

Chrousos, Charmandari; in collaboration with Cizza, Deuster, Dorn, Gold, Susman

The group has used corticotropin releasing hormone (CRH) to probe central mechanisms in patients with atypical, seasonal depression, the chronic fatigue/fibromyalgia syndromes, and the postpartum blues/depression syndromes. The patients' responses to CRH are indicative of mild, sustained hypocortisolism of central nervous system etiology, suggesting the existence of a spectrum of diseases characterized by hypofunction of the central stress system. These syndromes contrast with melancholic depression, panic anxiety, and anorexia nervosa, for which we earlier demonstrated hyperactivity of the CRH system. As we have shown, the behavioral similarities between patients with Cushing's syndrome and atypical depression probably stem from a similar pathologic decrease in CRH secretion. Recently, we demonstrated that preadolescent and adolescent girls with a history of sexual abuse exhibited an HPA axis response to CRH similar to that of patients with melancholic depression or recovering anorexics. The findings suggest that major stress in children may have long-term effects on the HPA axis accompanied by effects on the psyche. Indeed, we found dysthymia or depression in all the sexually abused children studied. In a social nonhuman primate model, we demonstrated effects of parental abuse not only on the HPA axis but also on growth.

Individuals who are carriers of a 21-hydroxylase deficiency, such as parents of patients with CAH, are presumably totally asymptomatic. We studied the mood and HPA axis of such subjects, testing the hypothesis that a compensatory elevation of CRH secretion would be associated with indices of increased anxiety. We found that, indeed, compensatory changes in the activity of the HPA axis are apparent in a standard CRH test and that these changes correlate positively with anxiety. As a group, the carriers had higher anxiety scores than appropriate controls. Studies in healthy normal volunteers demonstrated that, in middle age, the sleep centers in the brain are sensitized to CRH and glucocorticoids, a finding that explains the increasing vulnerability to stress- or caffeine-induced insomnia with advancing age. Studies in young and middle-aged patients with idiopathic insomnia showed that such patients have significant evening activation of their HPA axis and an overall activation of the axis that correlates positively with indices of poor-quality sleep, such as decrements in amount of deep sleep and increments in wakefulness. We also recently showed that antalarmin, a nonpeptide CRH antagonist that crosses the blood barrier, inhibits the stress-mimicking properties of CRH, including its effects on the HPA axis and behavior. The drug thus represents a new class of pharmacologic agents for the potential treatment of diseases characterized by CRH hypersecretion, such as melancholic depression, chronic anxiety disorder, anorexia nervosa, and idiopathic insomnia.

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Immune and reproductive CRH and role of CRH antagonists

Chrousos, Zoumakis; in collaboration with Dorn, Gold, Gravanis, Sano

We demonstrated that CRH is produced locally at sites of inflammation and has profound proinflammatory effects at an autocrine/paracrine level. CRH is a potent degranulator of mast cells, a phenomenon that can be inhibited by antalarmin, the nonpeptide CRH antagonist specific for type 1 receptors. The antagonist has marked systemic anti-inflammatory actions and ameliorates the disease in adjuvant-induced arthritis, a model of rheumatoid arthritis, suggesting that CRH antagonists may be useful in the treatment of inflammatory disorders in humans. CRH was also found in the ovary and endometrium, where it may participate in the inflammatory phenomena of ovulation, luteolysis, blastocyst implantation, and menstruation. Antalarmin blocked implantation in rats and labor in sheep, suggesting that CRH antagonists may have clinical applications in reproductive medicine.

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Cytokines and the inflammatory response in endocrine hyper- and hypo-secretion states

Chrousos; in collaboration with Elenkov, Gold, Sternberg, Vgontzas

Several cytokines, including interleukin 1, tumor necrosis factor-alpha (TNF-alpha), and interleukin-6 (IL-6), as well as several lipid mediators of inflammation, including platelet-activating factor and several eicosanoids, cause profound stimulation of the HPA axis, primarily by activating the CRH neuron and, through it, by suppressing and restraining the inflammatory/immune response. We recently studied IL-6 in humans and found that this cytokine is extremely potent in activating the HPA axis and that, at high doses, causes systemic vasopressin secretion. At all doses, it is not noxious, unlike TNF-alpha and interleukin-1, which cause hypotension and vascular leakage. We demonstrated that IL-6 is a potent stimulator of ACTH release in normal subjects and that catecholamines and glucocorticoids regulate IL-6 secretion. IL-6 levels are normal in patients with Cushing's syndrome but increase markedly after the surgical cure of hypercortisolism. The rebound elevation in circulating IL-6 levels immediately after surgical cure of hypercortisolism may be the cause of the systemic symptoms typical of the glucocorticoid withdrawal syndrome in these patients. Thus, IL-6 participates in an immune-endocrine feedback loop involving CRH, ACTH, and cortisol. We found that the stress hormones glucocorticoids and catecholamines inhibit the secretion of interleukin-12 and stimulate the secretion of IL-10 by monocytes/macrophages, leading to a shift from T helper 1– to T helper 2–directed immunity. We discovered that, in the transition from pregnancy to the postpartum period, the hormonal changes that take place cause a shift from T helper 2 to T helper 1, which explains the vulnerability of postpartum women to autoimmune disorders of the T helper 1 type, such as Hashimoto thyroiditis, multiple sclerosis, and rheumatoid arthritis.

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Leptin and cytokines in obesity, polycystic ovary syndrome, and sleep disorders

Chrousos, Ayala, Zoumakis; in collaboration with Bornstein, Vgontzas

We recently demonstrated that, in addition to leptin, human fat secretes large amounts of TNF-alpha and Il-6 and that the secretion of adipose tissue cytokines is regulated by food intake and the sympathetic nervous system. The secretion of these cytokines, e.g., IL-6, has a circadian rhythm that is influenced by sleep while circulating levels increase in proportion to BMI and are further elevated by visceral adiposity. Sleep deprivation is associated with elevations of daytime IL-6, whose levels correlate with somnolence and fatigue, particularly among patients with insomnia who, during the day, have elevated levels not only of cortisol but also of IL-6. Interestingly, IL-6 concentrations in plasma are proportional to body fat, and viscerally obese subjects have further elevations that correlate with sleep apnea measures. Women with polycystic ovary syndrome exhibit high cytokine and leptin levels and are at markedly increased risk for sleep apnea while girls with premature adrenarche show features of visceral adiposity such as high fasting leptin and insulin levels, predictors of later development of polycystic ovary syndrome and sleep apnea. The elevation of inflammatory cytokines in obesity and its correlation with many pathologic manifestations of this condition have led us to conduct clinical studies with biological antagonists of such cytokines.

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HYPERCORTISOLISM AND REPRODUCTIVE DISORDERS

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Over the past decade, we have made major contributions to the differential diagnosis of hypercortisolism. We developed the corticotropin releasing hormone (CRH) test and inferior petrosal sinus sampling (IPSS) as important diagnostic tools in the identification of pituitary adenomas causing Cushing's syndrome. However, the detection of Cushing's syndrome remains difficult, as does the localization of ectopic ACTH-producing tumors. We also evaluate the pathophysiology and potential new treatments for endometriosis and fibroids in women. These reproductive disorders are common, poorly understood, and lack optimal medical treatments.

Magnetic resonance imaging detection of corticotropinomas
Nieman; in collaboration with Oldfield, Patronas, Stratakis
Up to 70 percent of ACTH-producing pituitary adenomas (corticotropinomas) are not identified by the standard T1-weighted spin echo (SE) technique for magnetic resonance imaging (MRI). We hypothesized that the superior soft tissue contrast and thinner sections obtained with Spoiled Gradient Recalled Acquisition in the Steady State (SPGR) would improve tumor detection. We compared the diagnostic utility of SE and SPGR MRIs in a prospective study of 16 children (seven to 18 years) evaluated for pituitary tumors and 34 adults (to 67 years) admitted for jugular venous sampling. Testing included inferior petrosal sinus sampling if the SE MRI was normal. Coronal MR images were obtained on a 1.5 T scanner using gadolinium. We compared independent MRI reviews conducted by two radiologists, with tumor location recorded by a neurosurgeon at resection. Cushing's disease (CD) was confirmed by postoperative hypocortisolism and/or histopathology. Compared with SE for the detection of surgically identified tumor, SPGR had better sensitivity (47 versus 74 percent) but a lower specificity (20 to 33 percent versus 33 to 50 percent) because of a higher false positive rate (2 to 4 percent). The positive predictive value (PV) was similar for both techniques (90 to 95), but the negative PV was better for SE (4 versus 10) while diagnostic accuracy was better for SPGR (74 versus 48). We conclude that SPGR is superior to SE for detection of ACTH-secreting pituitary tumors. However, the higher false positive rate and lack of data about SPGR results in normal subjects suggest that imaging alone should not be used for the diagnosis of CD.

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Localization of ectopic ACTH-secreting tumors

Nieman; in collaboration with Pacak

Imaging studies are the cornerstone for tumor localization in patients with CD caused by ectopic adrenocorticotropin hormone (ACTH) secretion (EAS). Computed tomography and magnetic resonance imaging are used most commonly to localize the source of EAS. However, in 30 to 50 percent of patients with EAS, the source of ACTH secretion cannot be found despite repeated studies. Up to half of these patients do not respond to medical therapy for hypercortisolism and must undergo bilateral adrenalectomy with life-long replacement therapy. Thus, there is a need for improved imaging techniques to identify ACTH-secreting tumors.

Nuclear medicine techniques enable *in vivo* imaging of physiological and pathophysiological processes, and among these techniques, positron emission tomography studies are increasingly used in oncology. Ongoing studies evaluate whether [^{18}F]-fluorodeoxyglucose whole-body scanning or scintigraphy with the somatostatin analog [^{111}In-DTPA-D-Phe]-pentetreotide) may be useful in the detection of these tumors.

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Jugular venous sampling for the differential diagnosis of ACTH-dependent Cushing's syndrome

Nieman, Ilias; in collaboration with Oldfield, Pacak

In current clinical studies, we are evaluating the effectiveness of internal jugular vein (IJV) sampling as a substitute for the more invasive and technically demanding inferior petrosal sinus (IPS) sampling to distinguish patients with CD from those with ectopic ACTH syndrome. Preliminary data in 65 patients with surgically proven CD and in 13 patients with known or occult ectopic ACTH secretion showed a sensitivity of 83 percent for IJV sampling and 92 percent for IPS sampling; all patients with ectopic ACTH secretion were correctly identified. Further validation of this diagnostic procedure would allow local medical centers without expertise in IPSS to localize directly the source of ACTH hypersecretion in ACTH-dependent hypercortisolism, whereas at present only a few highly specialized centers offer the IPSS test.

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Quality-of-life assessment in Cushing's syndrome

Nieman; in collaboration with Nansel

We are now exploring quality-of-life issues in patients before and after successful treatment of Cushing's syndrome. We use the entire SF-36 questionnaire developed by John Ware and colleagues; the questionnaire has been validated for use in healthy individuals and various disease states. Patients also complete a symptom checklist and a questionnaire about recurrence, satisfaction with treatment, current treatment, and demographics. Initial analysis suggests that patients with active Cushing's syndrome have great impairment in physical activities and cognitive function and that they limit social activities. Patients also demonstrate significant but less profound abnormalities in their sense of well-being and general health perception compared with the general population. Preliminary evaluation of patients after treatment suggests improvement in these areas, but some patients do not regain scores similar to the general population for as long as five years after treatment, pointing to significant long-term impairment of quality of life in some individuals.

Estrogen receptors in normal endometrium and endometriosis

Nieman, Hearns-Stokes; in collaboration with Mayers, Segars, Zahn

We characterized the spatial and temporal localization of estrogen receptor (ER) alpha and beta in the endometrium and endometriosis explants of women with pelvic pain from endometriosis. Samples were obtained during laparoscopic laser excision surgery. Immunohistochemical staining for ER alpha showed nuclear localization that was greatest in the epithelium during the proliferative phase and that decreased during the late secretory phase. ER beta showed a similar nuclear and epithelial cell preference, but the intensity of ER beta immunostaining increased during the secretory phase. ER beta increased in endometriotic explants.

Raloxifene as an adjunctive treatment for endometriosis

Nieman, Lopez, Stratton, Sinaii, Nahari, Zhu, Nilson; in collaboration with Azumi, Chow, Daly, Karp, Koziol, Merino, Reynolds, Wesley, Winkel*

We also are conducting a randomized, double-blind placebo-controlled study of surgery with or without raloxifene (Evista®) for the treatment of pain from endometriosis. To date, we have examined the utility of MRI in diagnosing endometriosis and correlated histopathology with laparoscopic findings. Our analysis suggests that MRI has limited diagnostic utility in a cohort of women with pelvic pain because it has a low detection rate of biopsy-proven endometriosis lesions and is relatively insensitive in determining whether an individual woman has endometriosis. In comparing surgical and histopathologic findings, only about 70 percent of endometriosis lesions seen at surgery are proven by biopsy.

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CDB-2914 as a potential therapeutic agent for fibroids

Nieman, Nahari, Nilson; in collaboration with Armstrong, Catherino, Leppert, Premkumar, Segars

We have investigated the effect of single doses of the progestin receptor modulator CDB-2914 on the menstrual cycle in women and showed that doses of 100 or 200 mg retard folliculogenesis and precipitate menses in the follicular and luteal phases, respectively. Ongoing studies are evaluating whether the agent may shrink fibroid size and use gene arrays to evaluate the pathophysiology of leiomyomata.

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DIAGNOSIS, LOCALIZATION, PATHOPHYSIOLOGY, AND MOLECULAR BIOLOGY OF PHEOCHROMOCYTOMA

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We are conducting patient-oriented, conceptually innovative research on the etiology, pathophysiology, diagnosis, prognosis, and treatment of pheochromocytoma and other neuroendocrine tumors. Projects include not only translational research (applying basic science knowledge to clinical diagnosis, pathophysiology, and treatment) but also "reverse translation research," in which appreciation of clinical findings leads to new concepts that basic researchers can pursue in the laboratory. The long-range goal is to develop new and improved approaches in the diagnosis and treatment of pheochromocytoma. As an initial step toward this goal, studies focus on molecular and genetic mechanisms that may elucidate the bases for predisposition to develop pheochromocytomas and for expression of different neurochemical phenotypes and malignant potentials of these tumors. The unit also focuses on new imaging approaches based on 6-[^{18}F]fluorodopamine positron emission tomographic (PET) scanning and new biochemical diagnostic criteria based on measurement of plasma metanephrines and a novel clonidine test. Our research team will also be conducting clinical studies related to treatment of malignant pheochromocytoma by using ^{131}I-metaiodobenzylguanidine.

Biochemical diagnosis of pheochromocytoma
Pacak; in collaboration with Eisenhofer, Lenders, Linehan, Walther
The diagnosis of pheochromocytoma typically requires confirmation by several tests, perhaps the most important of which is biochemical evidence of excessive catecholamine production by the tumor. Such evidence usually derives from measuring catecholamines and certain catecholamine metabolites in urine or plasma. However, the catecholamines norepinephrine and epinephrine are also produced by sympathetic nerves and the adrenal medulla and are thus not specific to pheochromocytoma. Sometimes pheochromocytomas may be biochemically "silent," not producing catecholamines in amounts sufficient to obtain a positive biochemical test result or secreting catecholamines episodically; between episodes, catecholamine levels may be normal. Moreover, due to the low prevalence of pheochromocytoma in the tested population and inadequate specificity of biochemical tests, positive results in patients without pheochromocytoma are a common and troublesome occurrence. We have developed a biochemical test involving measurements of plasma free normetanephrine and metanephrine, respective metabolites of norepinephrine and epinephrine, which, in contrast to catecholamines, are produced continuously and independently of exocytotic catecholamine release.

We carried out a large prospective multicenter cohort study of 214 patients in whom the diagnosis of pheochromocytoma was confirmed and of 644 patients who were determined not to have the tumor.

Sensitivities of plasma free and urinary fractionated metanephrines were higher than those for plasma catecholamines, urinary catecholamines, urinary total metanephrines, and urinary vanillylmandelic acid. Specificity was highest for urinary vanillylmandelic acid and urinary total metanephrines; intermediate for plasma free metanephrines, urinary catecholamines, and plasma catecholamines; and lowest for urinary fractionated metanephrines. Currently, we are also attempting to determine the diagnostic utility of measurements of plasma free metanephrines in patients with metastatic pheochromocytoma before and after treatment.

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Molecular genetic basis of tumorigenesis in familial and sporadic benign and malignant pheochromocytomas

Brouwers, Ohta, Koch, Pacak; in collaboration with Breza, Eisenhofer, Ksinantova,, Kvetnansky, Linehan, Walther, Zhuang*

According to Knudson's well-established "two hit" hypothesis of hereditary tumor development, inactivation of both copies of a tumor suppressor gene is required to promote tumor growth. In contrast, the concept of tumorigenesis for oncogenes concerns mutation in the respective oncogene on one allele to be sufficient. Our collaborators from NINDS have recently proposed a new model of oncogene-driven tumor initiation. The model suggests overrepresentation of the mutated *c-met* oncogene as an important mechanism of hereditary papillary renal cell tumorigenesis. Homology between *MET* and *RET* led us to hypothesize that tumor formation in patients with multiple endocrine neoplasia type 2 (MEN 2)–associated tumors might develop by a similar mechanism. In this syndrome, which mainly consists of pheochromocytoma and medullary thyroid carcinoma, patients with a *RET* germline mutation develop tumors as early as in the first month of life and as late as in the seventh decade. In a series of experiments headed by Drs. Koch and Zhuang, we found that duplication of the mutated *RET* allele in trisomy 10 may represent a possible mechanism of tumorigenesis in MEN 2-associated pheochromocytomas. However, some of the pheochromocytomas did not reveal overrepresentation of mutated *RET*. Instead, the tumors had lost the wild-type *RET* allele, also leading to a dominant effect of mutant *RET*. This allelic imbalance between the mutant and wild-type *RET* allele could also be demonstrated in MEN 2-associated medullary thyroid carcinoma and in the TT cell line. In this cell line, we did not find trisomy 10 or loss of the wild-type *RET* allele as mechanisms of overrepresentation of mutant *RET* but, instead, a tandem duplication of *RET* as a third mechanism for imbalance between mutant and wild-type *RET*. Some of these MEN 2–associated tumors, however, did not reveal mutant and wild-type *RET* imbalance. We therefore searched for other mechanisms of a "second hit" in these tumors and found somatic *VHL* gene deletion and mutation. We are also investigating genetic mechanisms that may be responsible for a malignant potential of pheochromocytoma. For example, we are conducting experiments using gene expression profiling and proteomics to find out which genes and proteins may distinguish malignant pheochromocytomas from benign ones and to search for new markers of malignant pheochromocytoma.

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Imaging modalities in the evaluation of patients with pheochromocytoma

Pacak; in collaboration with Carrasquillo, Chen, Reynolds

Pheochromocytoma constitutes a form of surgically curable hypertension, and failure to diagnose and localize the tumor can result in sudden, unexpected, and potentially lethal complications. Computed tomography and magnetic resonance imaging have good sensitivity but poor specificity, and commonly available nuclear imaging modalities, such as ^{131}I-metaiodobenzylguanidine scintigraphy, have high specificity but limited sensitivity. Positron emission tomographic scanning, which uses short-lived positron-emitting radionuclides, allows administration of large tracer doses and results in high count density and thus better resolution than conventional single-photon emitters used in nuclear medicine. 6-[^{18}F]-Fluorodopamine, a sympathoneural imaging agent developed in the NIH intramural research program, is a positron-emitting analog of dopamine. In catecholamine-synthesizing cells including pheochromocytoma cells, 6-[^{18}F]fluorodopamine is transported actively and avidly by both the plasma membrane norepinephrine transporter and intracellular vesicular monoamine transporter.

Our results showed that, in patients with pheochromocytoma (including metastatic disease), 6-[^{18}F]-fluorodopamine PET scanning could detect and localize pheochromocytomas with high sensitivity. In patients in whom the diagnosis of pheochromocytoma is considered but excluded because of negative biochemical results, 6-[^{18}F]-fluorodopamine PET scans are consistently negative. Furthermore, our results showed that 6-[^{18}F]-fluorodopamine was superior to ^{131}I-metaiodobenzylguanidine in diagnostic localization of benign and metastatic pheochromocytoma. Currently, we are comparing the sensitivity and specificity of 6-[^{18}F]-fluorodopamine PET scanning with ^{123}I-metaiodobenzylguanidine scintigraphy and Octreoscan in the diagnostic localization of malignant pheochromocytoma.

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Mechanisms that link different pheochromocytoma tumor cell phenotypes and clinical presentation of disease to specific underlying mutations

Pacak, Brouwers; in collaboration with Eisenhofer, Zhuang

Our recent results indicated that pheochromocytomas from patients with von-Hippel Lindau (VHL) syndrome and MEN 2 display distinct neurochemical and histopathological phenotypic features accompanied by differences in the clinical presentations of the disease. Patients with MEN2 and pheochromocytoma have a high incidence of paroxysmal attacks and exhibit more robust adrenergic and hemodynamic responses to glucagon than do patients with VHL disease and pheochromocytoma. Pheochromocytomas from patients with VHL disease showed a distinctly noradrenergic phenotype, i.e., the tumor produces norepinephrine specifically, while those from patients with MEN2 showed an adrenergic phenotype, i.e., the tumor also produces epinephrine. These phenotypic variations result from differences in expression of certain genes, such as that encoding phenolethanolamine-N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine. We plan to relate these results and neurochemical analysis of tumor catecholamine content to the underlying germline and somatic mutations. Morphologic studies in conjunction with genetic analysis will attempt to separate the tumors into morphologically and genetically distinct subsets. Follow-up studies combining laser-capture microdissection with differential display or microarray analysis will trace back phenotypic differences in tumors to underlying differences in gene expression and, ultimately, to the basic somatic or germline mutations responsible for the tumor.

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Chromaffin and pheochromocytoma cell cultures and animal model of metastatic pheochromocytoma

Pacak, Brouwers, Ohta, Nave; in collaboration with Alesci, Tischler

There is no known effective treatment for malignant pheochromocytoma. Developing effective treatments requires a means to test new drugs for their ability to kill tumor cells selectively. New therapeutic options, such as gene therapy and immunotherapy, should also be considered. This project is designed to establish human pheochromocytoma cell lines to be used as *in vitro* models for testing radiotherapeutic or chemotherapeutic compounds, genetic manipulations, or vaccines that could be applied to the treatment or prevention of metastatic pheochromocytoma and other tumors of neural crest origin. We are identifying potential radiotherapeutic or chemotherapeutic drugs that might bind to or be taken up by and concentrated in the cells, oncolytic viruses or viral vectors for gene therapy, or proteins expressed specifically in pheochromocytoma tumor cells; determining the cytotoxic efficacy of the drugs or treatments; verifying that the drugs or treatments are not toxic to other cell lines; and developing new clinical research protocols to test the efficacy of the identified potential treatments of pheochromocytoma, especially malignant or unresectable pheochromocytoma. To generate an animal model of metastatic pheochromocytoma, we plan to give mice subcutaneous, intraperitoneal, and intravenous injections of both human and mouse pheochromocytoma cells and screen for the development of metastatic lesions in the lymph nodes, lungs, liver, and spleen. The metastatic lesions will be serially passaged and screened to select for subclones of high metastatic potential. We will follow the animals for tumor growth, measure blood catecholamine and metanephrine levels as surrogate markers for tumor burden, and then image tumors by using Atlas scanner. Tumors that develop in the mice will be removed for subcloning and used for genetic testing.

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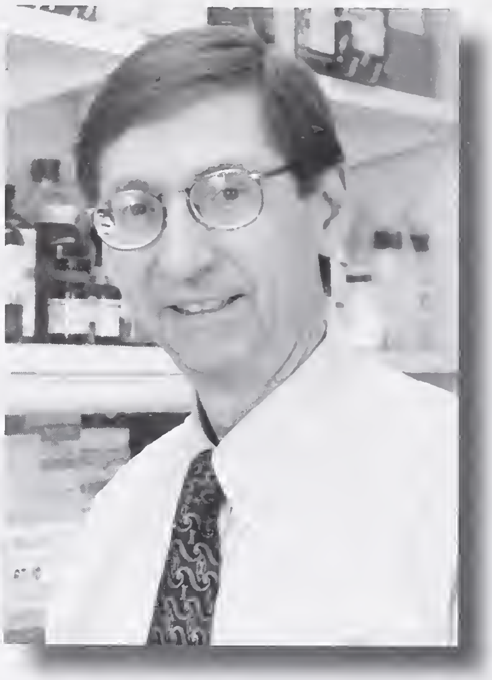

The objective of the unit is to provide clinician scientists with expertise in clinical and basic research relating to reproductive disorders and diseases. To that end, the unit supports the multi-institutional clinical training program in Reproductive Endocrinology and Infertility jointly sponsored by NICHD, the Uniformed Services University of the Health Sciences, and Walter Reed Army Medical Center. The unit conducts both basic and clinical research studies of importance to reproduction.

Uterine fibroids

Segars, Catherino, Leppert; in collaboration with Nieman, Tsibris

Uterine fibroids, also known as leiomyomata, are common benign tumors of the womb that may lead to excessive menstrual bleeding or infertility. Fibroids are three times more prevalent in women of African American heritage than in Caucasian women. It is estimated that up to 22 percent of African American women will obtain surgical treatment for fibroids. Billions of U.S. health care dollars are spent annually on treatment for uterine fibroids, but the cause of this common reproductive disorder remains unknown.

In the past year, basic science studies in our unit have used the powerful genetic method of cDNA arrays in an effort to identify genes involved in fibroid growth and development. In collaboration with John Tsibris, our approach has allowed us to examine up to 33,000 candidate genes for involvement in fibroid development. Using reverse-transcriptase PCR, real-time PCR, immunohistochemistry, and immunoblot experiments, we have confirmed the usefulness of the arrays to identify genes either over- or underexpressed in fibroids. The studies have focused attention on roughly 300 genes that appear to be differentially expressed in fibroids. Interestingly, the abnormalities in gene expression appear to be shared between fibroids obtained from different women. One emerging theme appears to be that fibroid development involves genes that regulate production of extracellular matrix; studies are planned to explore this possibility.

We have initiated clinical translational studies on uterine fibroids in collaboration with the team led by Lynnette Nieman in the branch. The goal is to examine new approaches to medical treatment for uterine fibroids.

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Mechanism of estrogen action

Segars, Tiulpakov, Mayers; in collaboration with Driggers, Westphal

Estrogen is of fundamental importance to many reproductive processes in women, in addition to its suspected role in breast and uterine cancer. In the past year, we have continued our basic science studies of the role of the *Brx* protooncoprotein in estrogen action. During the past year, studies conducted in collaboration with Heiner Westphal and Paul Driggers suggest that *Brx* may influence critical developmental processes and may regulate hormone receptors in addition to the estrogen receptor. The studies extend and support our finding that *Brx* augments estrogen action through important cytoplasmic signaling cascades, which, for the estrogen receptor beta, involve p38 mitogen–activated protein kinase. Future studies are directed to the *in vivo* role of *Brx* in reproduction and development.

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Infertility

Segars, Armstrong, Catherino, Neithardt, Parker

Ten percent of U.S. couples face the problem of infertility. Billions of U.S. health care dollars are spent annually on treatment of this condition and complications arising from treatments such as multiple births. As an integral part of the clinical fellowship in Reproductive Endocrinology, we have continued to examine causes of infertility. In addition, we continue to develop strategies to reduce multiple births and to identify factors that are positively or negatively associated with pregnancy in couples undergoing treatment.

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Premature birth is the leading cause of perinatal mortality and morbidity worldwide. The Perinatology Research Branch has defined preterm labor as a syndrome and determined that at least 25 percent of all preterm births are born to women with subclinical intrauterine infection. Moreover, we have provided evidence that many premature neonates are critically ill before birth and proposed that the onset of premature labor has survival value in the context of intrauterine infection. We aim to understand the pathophysiology of premature labor and delivery, particularly in the context of intrauterine infection and inflammation. This year, we conducted several studies to explore the role of antibiotic administration in the treatment of intrauterine infection and the value of assessing the concentration of antimicrobial proteins in the detection of intrauterine infection and inflammation. After premature birth, congenital anomalies are the second leading cause of perinatal mortality in the United States. This year, we used four-dimensional ultrasonography for the evaluation of the fetal heart with spatio-temporal image correlation and 3-D ultrasound to quantitate fetal lung volume measurements.

Antimicrobial factors in the amniotic fluid of normal women and those with preterm labor and premature rupture of membranes

Espinoza, Chaiworapongsa, Romero, Edwin

The amniotic cavity is considered a sterile compartment, presumably owing to the innate immune system, and includes the cervical epithelium, cervical mucus plug, chorioamniotic membranes, and cellular components of the decidua, amnion, and chorion, including neutrophils, macrophages, NK cells, and trophoblast. Although intact chorioamniotic membranes are thought to protect against microbial invasion of the cavity (MIAC), this physical barrier alone cannot be responsible for the sterile nature of amniotic fluid in normal pregnancy because bacteria can cross the intact membranes. Indeed, patients with intact membranes can have MIAC, as has been demonstrated in patients in the midtrimester of pregnancy with preterm labor and intact membranes, or even spontaneous labor at term. This suggests that, in addition to the physical barrier, other mechanisms must operate to prevent bacterial proliferation within the amniotic cavity. Antimicrobial peptides, part of the innate limb of the immune response, have been identified in plants, insects, and all vertebrates examined thus far. Neutrophils contain several antimicrobial peptides, including neutrophil defensins (human neutrophil peptides [HNP] 1, 2, and 3), bactericidal/permeability-increasing protein (BPI), and calprotectin (MRP8/14). The purpose of this study was to determine if amniotic fluid contains immunoreactive antimicrobial peptides (HNP 1-3, BPI, and MRP8/14) and whether their concentration changes with MIAC, parturition, and premature rupture of membranes (PROM). We conducted a series of studies that yielded the following: (1) intraamniotic infection is associated with a significant increase in amniotic fluid concentrations of HNP1-3, BPI, and calprotectin in both women with preterm labor and intact membranes and women with preterm PROM; (2) preterm PROM is associated with a significant increase in amniotic fluid concentrations of HNP 1-3, BPI, and calprotectin; (3) preterm parturition is associated with a significant increase in amniotic fluid concentrations of HNP 1-3, BPI, and calprotectin while term parturition is associated with a significant increase in amniotic fluid concentrations of immunoreactive HNP 1-3; and (4) among patients with preterm labor and intact membranes, elevation of amniotic fluid HNP 1-3, BPI, and calprotectin concentrations was associated with intraamniotic inflammation, histologic chorioamnionitis, and short interval to delivery.

Espinoza J, Chaiworapongsa T, Romero R, Edwin S, Rathnasabapathy C, Gomez R, Bujold E, Camacho N, Kim YM, Hassan S, Blackwell S, Whitty J, Berman S, Redman M, Yoon BH, Sorokin Y. Antimicrobial peptides in amniotic fluid defensins, calprotectin and bacterial/permeability-increasing protein in patients with microbial invasion of the amniotic cavity, intra-amniotic inflammation, preterm labor and premature rupture of membranes. *J Matern Fetal Neonatal Med* 2003;13:2-21.

The effect of antibiotic therapy on intrauterine infection–induced preterm parturition in rabbits

Romero, Chaiworapongsa, Espinoza

Microbial invasion of the amniotic fluid cavity and subclinical intrauterine infection have been implicated as mechanisms responsible for preterm labor and delivery. Consequently, investigators have examined whether the administration of antibiotics to women in preterm labor with intact membranes and preterm premature rupture of membranes can prolong pregnancy and improve neonatal outcome. The results from randomized clinical trials and meta-analysis are conflicting. The Cochrane Review concluded that routine use of antibiotics is not effective in preventing preterm parturition with intact membranes. In contrast, animal experiments suggest that, in some instances, antibiotic administration can reduce intra-amniotic bacterial load and preterm delivery as well as improve neonatal survival. One possible explanation for antibiotics' ineffectiveness in humans is that medication must be administered early to be effective and that, when patients present with clinical preterm labor and intraamniotic infection, it is too late to down-regulate the inflammatory response and thus antimicrobial therapy cannot prevent preterm parturition and/or fetal damage. We used a rabbit model of ascending uterine infection to assess whether there is a time-dependent relationship between antibiotic administration and the prevention of preterm parturition. We tested whether rabbits inoculated with *E. coli* without antibiotic treatment would deliver prematurely. The median inoculation-to-delivery interval was significantly shorter in the infected group than in the control group (36.3 hours [15 to 76.5] versus 219 hours [173 to 240]). Antibiotic administration within 12 hours, but not after 18 hours, of inoculation increased the duration of pregnancy (by reducing the rate of preterm delivery) and neonatal survival (0 versus 71 percent). We found that antibiotic administration can prolong pregnancy and reduce perinatal mortality after an ascending intrauterine infection only if administered early (less than 12 hours) after microbial inoculation. Whether this approach could also reduce neonatal morbidity remains to be determined. The importance of this observation is that it points to the need for the rapid and early identification of intraamniotic infection in humans.

Maternal serum of women with preeclampsia reduces trophoblast cell viability: evidence for an increased sensitivity to Fas-mediated apoptosis

Chaiworapongsa, Romero; in collaboration with Mor

Preeclampsia, or toxemia of pregnancy, has been attributed to the presence of a circulating "toxin" that disappears from peripheral blood after delivery of the placenta. However, the presence, nature, and effects of this toxin have eluded characterization. Increased trophoblast apoptosis has been observed in the placenta of women with preeclampsia, and it is possible that this biological phenomenon is important for the genesis of the disease and is mediated through a soluble factor(s) present in maternal blood. We conducted a study to determine whether serum from women with preeclampsia changes trophoblast viability. We cultured H8 trophoblast cells with serum obtained from normal pregnant women and patients with preeclampsia. We determined cell viability by Cell Titer 96 Assay and Fas sensitivity by treating the cells with an anti-Fas antibody or a blocking anti–Fas Ligand antibody. Serum from normal pregnant women did not affect trophoblast cell viability. In contrast, serum from preeclamptic women reduced trophoblast viability, an effect that was enhanced by treatment with an anti-Fas antibody and reversed by the treatment with a blocking anti–Fas Ligand antibody. We conclude that serum from women with preeclampsia induces cytotoxicity of the first-trimester trophoblast cell line (H8), an effect that appears to be related to changes in trophoblast sensitivity to Fas-mediated apoptosis. The findings suggest that a factor present in maternal blood of patients with preeclampsia may have a role in the genesis of the syndrome. Future studies will aim at determining whether this factor is present before the development of the disease.

Neale D, Demasio K, Illuzi J, Chaiworapongsa T, Romero R, Mor G. Maternal serum of women with preeclampsia reduces trophoblast cell viability evidence for an increased sensitivity to Fas-mediated apoptosis. *J Matern Fetal Neonatal Med* 2003;13:39-44.

Four-dimensional ultrasonography of the fetal heart with spatio-temporal image correlation

Chaiworapongsa, Espinoza, Romero

Congenital heart disease is the leading cause of birth defect–related infant mortality, with a prevalence ranging from four to 11 per 1,000 births. Prenatal diagnosis remains a major challenge. Four-dimensional (4-D) ultrasonography is a technology that couples motion with three-dimensional (3-D) imaging. Spatio-temporal image correlation (STIC) is a recent advance that allows dynamic multiplanar slicing and surface rendering of heart anatomy. Fetal heart volumes are acquired with a single automated sweep of the transducer. Spatial and temporal information is combined to display dynamic images that can be extracted from volume data sets. Standardized viewing planes, such as the one recommended by the American Institute of Ultrasound in Medicine, can be analyzed. In addition, planes not routinely feasible with two-dimensional (2-D) ultrasonography can be generated. This year, we reported using STIC to examine the fetal heart and for the diagnosis of congenital anomalies. We obtained volume data sets that allowed us to demonstrate the cardiac chambers, moderator band, interatrial and interventricular septae, atrioventricular valves, pulmonary veins, and outflow tracts. With the use of a reference dot to navigate the four-chamber view, we were able to study intracardiac structures simultaneously in three orthogonal planes. We used the same volume data set for surface rendering of the atrioventricular valves. The aortic and ductal arches were best visualized when the original plane of acquisition was sagittal. Volumes could be interactively manipulated to visualize both outflow tracts simultaneously as well as the aortic and ductal arches. We generated novel views of specific structures. Dynamic multiplanar slicing and surface rendering of the fetal heart are feasible with STIC technology. One good-quality volume data set, obtained from a transverse sweep, can be used to examine the four-chamber view and the outflow tracts. This novel method may assist in the evaluation of fetal cardiac anatomy.

Goncalves LF, Lee W, Chaiworapongsa T, Espinoza Dorado J, Schoen ML, Falkensamer P, Treadwell M, Romero R. Four-dimensional ultrasonography of the fetal heart using spatio temporal image correlation. *Am J Obstet Gynecol* 2003;189:1792-1802.

Three-dimensional color power imaging of fetal hepatic circulation

Kalache, Romero, Chaiworapongsa, Espinoza; in collaboration with Lee

The anatomy and physiology of the fetal hepatic system are complex. Yet their evaluation is of considerable importance in assessing the hemodynamic status of a critically ill fetus. Furthermore, congenital anomalies of this circulatory system are relatively common and difficult to identify with conventional 2-D imaging techniques. We set out to determine how automated 3-D power Doppler ultrasonography defines the normal anatomy of the fetal portosystemic and umbilical venous systems and identifies fetal hepatic vascular anomalies. In a prospective study, we imaged the hepatic and umbilical venous systems in 390 fetuses with 2-D ultrasonography and color and spectral Doppler imaging and observed vascular abnormalities in eight of the fetuses. The anomalies were absent ductus venosus (n=4); direct connection between the umbilical vein and the right atrium (n=2); and direct connection between the umbilical vein and the inferior vena cava (n=2). The study demonstrated that 3-D color power Doppler ultrasonography can be used to image normal fetal hepatic and portal circulation and to identify anomalies of the fetal portosystemic and umbilical venous systems.

Kalache K, Romero R, Goncalves LF, Chaiworapongsa T, Espinoza J, Schoen ML, Treadwell MC, Lee W. Three-dimensional color power imaging of the fetal hepatic circulation. *Am J Obstet Gynecol* 2003;189: 1401-1406.

Lee W, Kalache KD, Chaiworapongsa T, Londono J, Treadwell MC, Johnson A, Romero R. Three-dimensional power Doppler ultrasonography during pregnancy. *J Ultrasound Med* 2003; 22:91-97.

Three-dimensional ultrasound fetal lung volume measurement: a systematic study comparing the multiplanar method with the rotational (VOCAL) technique

Kalache, Espinoza, Chaiworapongsa, Romero; in collaboration with Lee

Prenatal diagnosis of pulmonary hypoplasia is a major clinical challenge. The diagnosis is made at autopsy by demonstrating low lung weight and a low lung-to-body ratio. Several fetal biometric parameters have been used to predict the likelihood of lethal pulmonary hypoplasia of the newborn; however, nearly all parameters developed thus far are less than optimal. 3D ultrasound may improve the estimation of fetal organ size. In addition, rotational measurement of volume has now become possible through the recent introduction of a software tool named VOCAL (Virtual Organ Computer-Aided AnaLysis, 3D View). The software allows volume calculation by rotating the organ of interest around a fixed axis through a number of sequential steps. We studied 32 fetuses with a variety of conditions at risk for pulmonary hypoplasia. We acquired 3-D volume data sets of the fetal lungs by using a commercially available ultrasound system and separately calculated the right and left lung volumes by using VOCAL and the multiplanar technique. We determined the level of agreement between two independent observers in categorizing the 3-D volume data set as measurable or nonmeasurable and evaluated the interobserver and intermethod variabilities for both methods. The intermethod variability was excellent, demonstrating substantial agreement between the results of both approaches. Fetal lung estimation with VOCAL had a significantly higher interobserver variability than the multiplanar technique. Interobserver agreement in categorizing lung volume data sets as measurable or nonmeasurable was lower when VOCAL was used. Fetal lung volume measurements can be undertaken interchangeably by using the multiplanar technique or the rotational method with VOCAL. However, the latter was less reproducible (lower degree of agreement and significantly higher interobserver variability) than the former.

Kalache KD, Espinoza J, Chaiworapongsa T, Londono J, Schoen ML, Treadwell MC, Lee W, Romero R. Three-dimensional ultrasound fetal lung volume measurement: a systematic study comparing the multiplanar method with the rotational (VOCAL) technique. *Ultrasound Obstet Gynecol* 2003;21:111-118.

Genetic sonography: an option for women of advanced maternal age who are screen-negative following triple-marker maternal serum screening

Romero; in collaboration with DeVore

The prenatal detection of trisomy 21 is a major goal in prenatal care for families seeking antenatal diagnosis. Several approaches are available, including (1) routine offering of amniocentesis to women age 35 and over; (2) maternal serum screening and identification of patients at risk; and (3) a combination of the previous two with a sonographic examination of the fetus. Trisomy 21 is often associated with phenotypic abnormalities detectable with ultrasound. We conducted a study to determine whether conducting genetic ultrasound to patients 35 years and older who are screen-negative following maternal serum triple-marker screening is cost-effective and results in an increase in the detection rate of trisomy 21. We determined the detection rate and cost of detecting trisomy 21 in women 35 years and older, who were managed as follows: universal genetic amniocentesis; maternal-serum triple-marker screening followed by amniocentesis only in high-risk women (risk greater than 1:90); genetic ultrasound in women who were screen-negative; and genetic amniocentesis in patients with an abnormal genetic ultrasound. We concluded that offering genetic ultrasound followed by amniocentesis to patients 35 years and older who were originally screen-negative following triple-marker maternal serum screening for the diagnosis of trisomy 21 is cost-effective and results in a higher overall detection rate for trisomy 21.

DeVore GR, Romero R. Genetic sonography: an option for women of advanced maternal age who are screen-negative following triple-marker maternal serum screening. *J Ultrasound Med*, 2003; 22:1191-1199.

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RESEARCH IN PHYSICAL DISABILITIES

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The Physical Disabilities Branch (PDB) was established in part to accelerate the formation of a highly collaborative, multidisciplinary, technology and rehabilitation research network. We develop and apply advanced technologies that create new directions for our basic, translational, and clinical rehabilitation research in support of the transrehabilitation research domain model, which postulates that our ability to understand disablement rests on linking knowledge of the impairment, functional limitation, and physical disability rehabilitation research. These advanced technologies encompass a broad range that includes theoretical concepts, numeric models, computational methods, and instrumentation development. We use the technologies to conduct translational and clinical research in the following patient diagnostic categories: post-polio syndrome; stroke, rheumatoid arthritis/ osteoarthritis; neuropathy; myositis; cerebral palsy; cancer and spinal cord injuries; cystinosis; Sjögren's syndrome; multiple sclerosis; and dysphagia symptoms.

Induced acceleration analysis for rehabilitation

Kepple, Lohmann Siegel, Selbie, Stanhope; in collaboration with Neptune

By studying the contribution of net joint moments and individual muscle forces to the control of human movement and the performance of activities of daily living, we intend to link a physically challenged person's capabilities to his or her ability to perform activities of daily living. Measuring these contributions is critical for defining adaptive movement control strategies and understanding the process whereby the strategy of movement control is selected and implemented.

Earlier efforts to determine the contribution of net muscular joint moments and individual muscle forces to human movement control have been limited by the assumption that the relative contribution of muscular effort to the control of movement is proportional to the magnitude of the effort and independent of the position of body segments. A generalized coordinate formulation of the equations of motion shows that the assumption is incorrect and can lead to errors in interpreting human movement analysis data. We have extended existing gait analysis methodology by developing a mechanical linkage model that provides direct estimates of the influence a given joint moment or muscle force on the movement of all joints, body segments, and overall task performance.

Our research revealed that the generation of forward velocity during normal gait arises primarily from ankle joint plantar flexor push-off rather than through a controlled fall. We found that the ankle joint plantar flexor muscle group produces forward acceleration of the upper body during periods in which the same muscle group acts eccentrically. The literature suggests that deceleration of the lower extremity swing limb is an additional source of forward velocity generation during gait. However, our results indicate that the swing limb moments do not make a significant contribution to the forward acceleration of the upper body during normal walking. We examined the relative contribution of the lower extremity joint moments to the maintenance of upright posture (support) and found that, during the single limb support phase, ankle moments make the greatest contribution to support. During double limb support, the knee and hip moments of the forward positioned leg increase their contributions to support.

We also examined the relative sensitivity of the joint accelerations to the joint moments during flat-foot and found that the ankle, knee, and hip joint accelerations are almost twice as sensitive to moments generated at the knee than to the moments at any other lower extremity joint. During the heel-off phase of gait, the acceleration sensitivities at both the ankle and hip joints increase in response to moments at their own joint. It is clear from the data that there is significant redundancy in controlling the motion of the stance limb. Our findings explain, in part, the ability of persons with muscle weakness to compensate by using alternative movement control strategies. We further extended the model to estimate the source of mechanical energy exchange between segments and used it to determine the muscle groups responsible for the intersegment transfer of mechanical energy during gait, showing that the energy transfer depends on the sign of the joint moment rather than on the sign of the joint power and that pairs of net joint moments function in combination to balance energy flow through the leg and trunk segments during normal gait. We have further enhanced the induced acceleration analysis models to track the deformations in ankle-foot-orthosis (AFO). By first measuring the visco-elastic properties of the AFO and then applying our latest models, we are now able to track which muscle groups are working to bend the AFO and thus store energy in the device. We are then able to determine how much energy is released by the device during the later part of stance phase and where the released energy goes.

Future research will develop a seamless integration of the induced acceleration techniques with the C-Motion, Visual3D software. We have also started to develop a clinical biomechanical model to determine the capacity and, ultimately, the actual contribution of individual muscles to controlling motion during a functional task. We also intend to expand our analysis to evaluate the utility of other assistive technologies (e.g., instrumented walkers) in the control of human movement and performance of activities of daily living.

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Siegel KL, Kepple T, Stanhope SJ. Joint moment control of mechanical energy flow during normal gait. *Proceedings of the IV World Congress of Biomechanics*, 2002; published on CD.

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Ankle foot orthosis contributions to net ankle moments in gait

Nelson, Lohmann Siegel, Kepple, Halstead, Stanhope

A variety of disorders and diseases affecting muscle and nerve function can cause sufficient lower extremity weakness to affect gait. Some individuals may be unable to ambulate while others

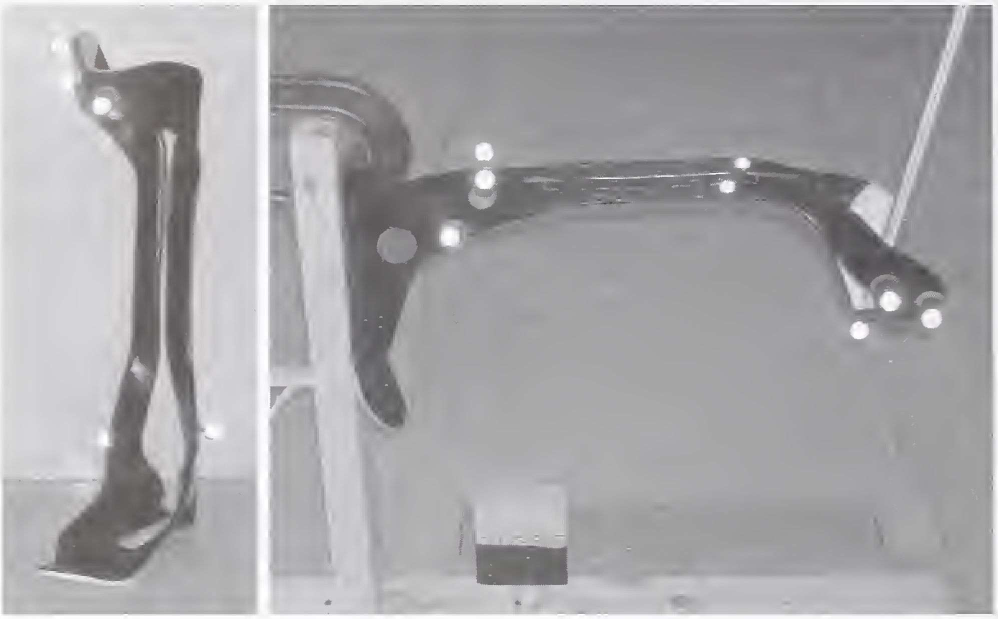

FIGURE 22.1

Dynamic carbon fiber AFO shown in neutral and bench testing positions

have limited ability to walk. Frequently, these individuals are prescribed orthotic devices to assist their ambulation. The financial impact is substantial, as Medicare expenditures for related prosthetics and orthotics reached $900 million in 2002. Yet, little is known of the assistance that advanced brace designs provide during functional tasks. Conventional AFOs are made of polypropylene and can deform in variable and unpredictable ways, hindering studies of the mechanical properties of the orthoses and the functional assistance they provide during walking. A new AFO design (Advanced Prosthetics and Orthotics Inc., Encinitas, CA) is made from continuous carbon fiber and consists of semirigid tibial cuff and footplate components connected by two deformable posterior longitudinal struts (see Figure 22.1). The materials and design of the new brace are much better suited to mechanical testing and modeling than older polypropylene devices.

By developing testing procedures to determine the mechanical properties of the brace and models that allow the brace's contribution to gait to be quantified, our goal is to evaluate the mechanical properties of the dynamic AFO, quantify the assistance it provides during gait, and evaluate the effect of the brace on lower extremity function during gait. We used a force platform and three-dimensional motion capture system in bench testing and to record kinetic and kinematic data from braced and unbraced walking trials for two subjects with post-polio syndrome. Bench testing established different spring stiffness relationships for each of the two subjects' AFOs. Using the spring model, we isolated the moment contributions from the AFO and the subjects' muscles during the walking trials for comparison with unbraced ankle moments.

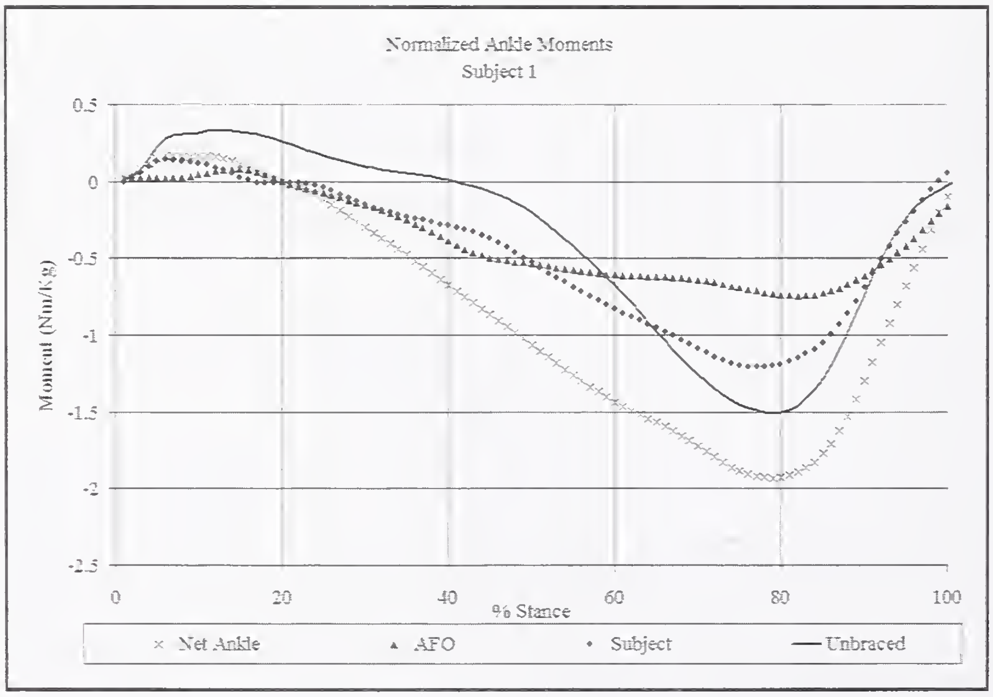

FIGURE 22.2

Subject 1 comparison of ankle moments for four test conditions: subject and AFO net ankle moment (Net Ankle); AFO contribution to net ankle moment (AFO); subject contribution to net ankle moment (Subject); and unbraced walking trial ankle moments (Unbraced).

In braced and unbraced walking trials, we noted minimal differences in walking speed and stride length for subject 1. In late stance, the peak subject ankle moment was reduced by 20 percent in the braced condition compared with the unbraced condition despite a 29 percent larger net ankle moment (see Figure 22.2). Therefore, the advanced AFO may benefit this particular subject by reducing the level of ankle joint muscle activity needed to maintain the same walking speed. In contrast, as compared with unbraced trials, subject 2 experienced increased speed and stride length when wearing the dynamic AFO. At the same time, subject 2 was able to generate a net ankle moment that was 100 percent greater than that without the brace while maintaining an identical subject-generated moment (see Figure 22.3). The results indicate that the increases in speed and stride length observed for the subject are solely attributable to increased moment generation capabilities from the AFO. This measurement capability is a first step toward quantifying the contributions from, and ultimately improving on, advanced assistive technologies.

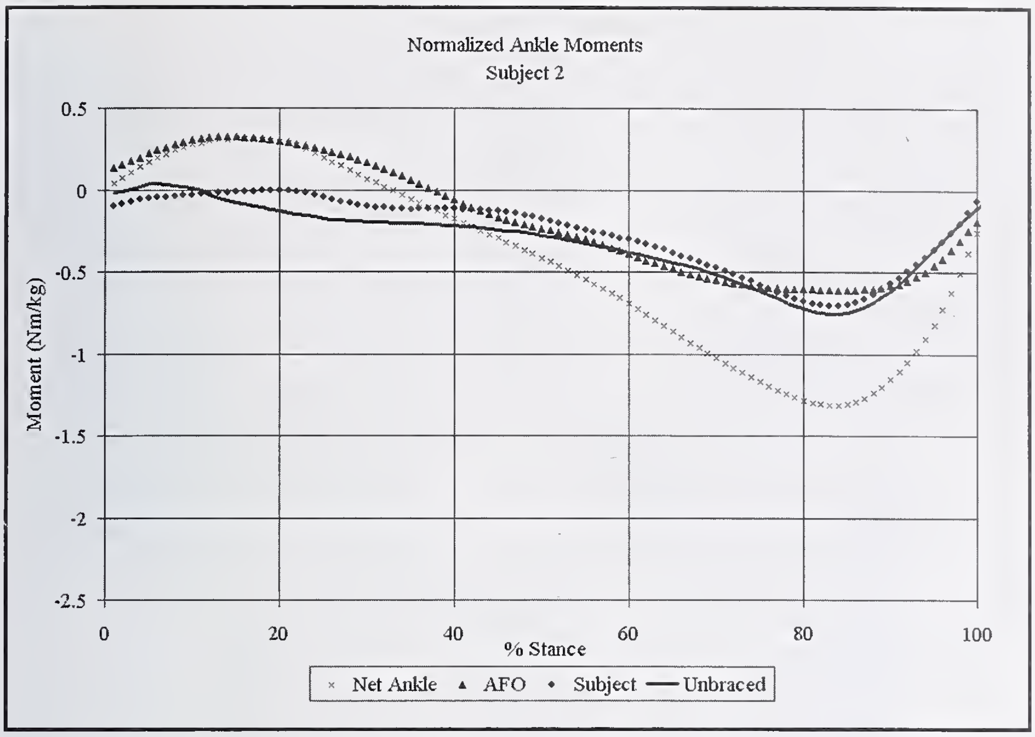

FIGURE 22.3

Subject 2 comparison of ankle moments for four test conditions: subject and AFO net ankle moment (Net Ankle); AFO contribution to net ankle moment (AFO); subject contribution to net ankle moment (Subject); and unbraced walking trial ankle moments (Unbraced).

Nelson KM, Kepple TM, Siegel KL, Halstead LS, Stanhope SJ. Ankle foot orthosis contribution to net ankle moments in gait. *American Society of Biomechanics*, Toledo, OH, September 2003; in press.

Movement visualization and analysis for rehabilitation

Kepple, Lohmann Siegel, Nelson, Bender, Selbie, Sheehan, Stanhope

An ongoing challenge for the Biomechanics and Biomedical Engineering (BBE) section is the continued development, enhancement, and maintenance of our powerful set of human movement analysis tools. Since 1988, the BBE section (formally part of the Biomechanics Laboratory) has been developing, with a rehabilitation focus, generalized computational tools for the analysis of human movement disorders. Historically, command line versions of the NIH software package have resided in the public domain and have been licensed to numerous facilities throughout the world under the material transfer agreement mechanism. The objective of the ongoing project is to create a powerful yet cost-effective software environment that provides clinicians, scientists, and engineers with access to existing PDB biomechanical modeling and human movement analysis methodologies and a software development infrastructure to support our new and emerging biomechanical modeling and analysis techniques. Figure 22.4 contains single-frame examples of the segment tracking, anthropometric modeling, and musculoskeletal reconstruction and analysis capabilities of algorithms.

To accomplish our goal, the PDB teamed with C-motion, Inc., and the Catholic University of America to convert the original NIH biomechanics software from a library of Fortran routines with a command line interface to an extensive collection of powerful routines based on Visual C++. The new library brings together several new computer programs (Visual3D, CALtester, and FPloc) that are analytically powerful, low-cost, and visually oriented and that can run on personal computers. Under this Small Business Innovation Research (SBIR) collaboration, the PDB retains unrestricted access to the extensive (source code) library of data management, analysis, and display routines. Such access provides an advanced platform on which to build future biomechanical modeling and

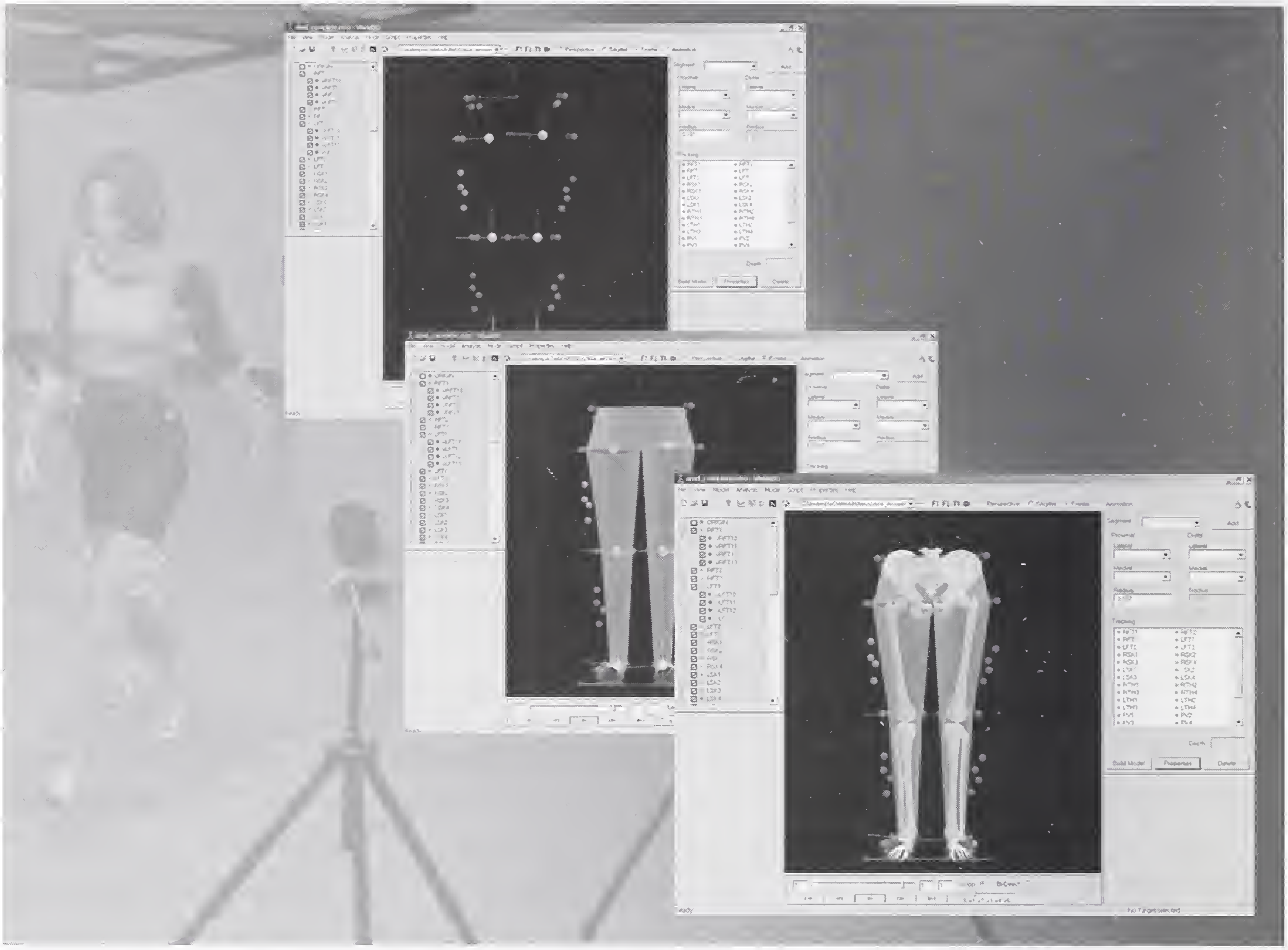

FIGURE 22.4

Panel containing a background image of the motion capture process and three panels depicting (from left to right) a set of lower extremity segment tracking targets, the application of a subject-specific anthropometric model, and the result of applying a scaled musculoskeletal model.

human movement analysis capabilities (e.g., the Induced Acceleration Analysis Initiative) and to launch several technology development initiatives (e.g., the Virtual Functional Anatomy Initiative and instrumented walker technologies).

Our past accomplishments include conversion of all primitive utilities associated with the preparation of data for biomechanical analyses to Visual C++ with significant enhancements to the automated event-determination algorithms, creation of the events storage structure, the development of a range class, and the design of a metrics data class. Furthermore, we have designed and implemented a universal viewer utility, an interactive graph utility, an enhanced data (C3D) file format, a method for the automated processing of motion data, a visual biomechanical model-building utility, and a report generation utility. We plan to continue to merge advancing modeling and human movement analysis technologies developed in the PDB with the Visual3D library, including induced acceleration analysis methods, dynamic MRI imaging data, instrumented walkers, and motion data derived from a proposed new three-dimensional universal movement assessment device. The execution of this highly collaborative, multidisciplinary, multiyear initiative has brought the most powerful human movement analysis techniques to scientists, clinicians, educators, and students worldwide on a cost-effective basis.

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Accuracy and reliability of movement analysis techniques

Stanhope, Nelson, Kepple, Lohmann Siegel, Selbie; in collaboration with Holden, Manal

The general purpose of this ongoing series of projects is to evaluate continually and improve the accuracy and reliability of biomechanical modeling and human movement analysis techniques so that the methods and findings will support the field's emerging quality assurance needs. Our past accomplishments include the development of a device and procedure for the automatic and accurate determination of the position and orientation of multiple force plate systems, a method developed by the BBE Section and implemented by C-motion, Inc., in the program FPLoc.

The percutaneous skeletal tracker (PST) is a device designed by Steven Stanhope to achieve minimally invasive skeletal fixation of kinematic targets to human limbs. The PST enables us to measure actual bone movement and compare it directly with motion measured simultaneously using surface-mounted targets. Recently, we used the method to determine the optimal method for attaching surface-mounted markers. We also found that optimal surface-mounted target attachment techniques are incapable of locating proximal tibial position with sufficient accuracy to measure strain on joint structures such as the anterior cruciate ligament.

We have developed a powerful technique for simultaneously evaluating the performance of all components of a clinical movement analysis laboratory. The Commission for Motion Laboratory, Inc., recently endorsed use of the technique by clinical movement analysis laboratories seeking accreditation. C-Motion, Inc., has implemented the method in the program CALtester, and Motion Lab Systems, Inc., is currently distributing the testing device.

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Virtual functional anatomy

Sheehan, Cohen, Rausch, Brant, Graham, Hunt, Li, Stanhope; in collaboration with Boden, Lu, Luo, Mohammed, Rebmann, Weston, Wirick, Yao

The Virtual Functional Anatomy (VFA) project is designed to fill the important knowledge gap that exists between the relationship of normal or impaired joint structure/function and the functional movement limitations associated with performing activities of daily living. Our current focus is on developing and ultimately validating a combined set of tools that will enable the accurate and precise measurement, analysis, and visualization of three-dimensional static and dynamic musculoskeletal anatomy (i.e., bone shape, skeletal kinematics, tendon and ligament strain, muscle force, and joint space). We plan to combine MRI imaging and analysis capabilities with a highly accurate, imaging-based measurement and analysis technique for the noninvasive quantification of complete joint anatomy and tissue dynamics during functional movements. Our effort will require the development of a method for creating 3-D digital images of loaded and moving joint tissues (bone, cartilage, and connective tissues) to reveal joint contact patterns and tissue loads. We plan to evaluate the variability of bone shape and the sensitivity of defined joint posture (translation and rotation of one bone relative to another) to osteo-based coordinate system definition. We intend to use these capabilities to document and evaluate the function of normal and impaired joint structures (e.g., ACL rupture and patellar tracking syndrome) under simulated conditions experienced during activities of daily living.

We have demonstrated the feasibility of studying musculoskeletal kinematics with cine-phase contrast (PC) and fast-PC MRI (the primary component of VFA) and determined the accuracy of Cine-PC MRI and, by extension, fast-PC MRI in the measurement of skeletal kinematics, in particular, the precision of Cine-PC and fast-PC MRI in the measurement of knee joint kinematics and patellar tendon strain during a leg extension task, under load. Work is ongoing to define static and dynamic measures of patellar tracking abnormalities as a potential source of patellofemoral (PF) pain and to determine which 3-D imaging sequence is the most accurate and precise in defining skeletal landmarks, cartilage thickness, and joint space profiles. We are developing a technique to define skeletal-based coordinate systems based on skeletal landmarks and to determine their sensitivity to small variations in landmark definition.

Our work involves three major areas of interest. First, we quantify healthy knee joint dynamics during loaded tasks that mimic functional activities of daily living. We then compare the resultant data with the knee joint dynamics of impaired subjects (e.g., patellar maltracking problems and anterior cruciate ligament loss). The second area is similar in that we are quantifying the 3-D kinematics of the bones of the ankle joint during loaded tasks mimicking functional activities of daily living. Third, we continue to make improvements in imaging time and data accuracy through algorithm and image sequence development.

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Association between subject functional status, seat height, and movement strategy in sit-to-stand performance

Mazzà, Stanhope; in collaboration with Benvenuti, Bimbi

Understanding the process and governing principles that link impairment to functional movement limitations and, ultimately, to disability is a fundamental goal of the rehabilitation and geriatric research fields. Impairments may cause the inability to perform specific body functions and result in disability when physiological reserve is markedly reduced and an individual cannot devise successful compensatory strategies. To assess the association between a subject's functional status and the role and effectiveness of compensatory movement strategies, we investigated an experimental sit-to-stand (STS) paradigm. The difficulty in performing the STS task is inversely related to the height of the chair seat. When the chair is lowered, the biomechanical demands increase and can reach levels that require compensatory movement strategies to complete the task successfully. We speculated that, when the demands become too great for any compensation to overcome, the individual will lose the ability to stand successfully.

Several strategies exist to assist an individual with the execution of an STS task: foot positioning, speed of execution, and trunk movements can be changed, and the use of the arms can be introduced. Subjects with impairments of the neuromusculoskeletal system create an overall compensatory movement strategy by simultaneously implementing a set of basic compensations. The effectiveness of the resulting effort is a function of the subject's level of impairment, the effectiveness of the compensatory movement strategy, and the overall difficulty of the movement task. We think it is important to understand the interaction between the different determinants of the STS task and therefore focused on the analysis of the association among the individual's functional status, the difficulty of the movement task, and the effectiveness of compensatory movement strategies.

Our study involved a sample (n=131) of the elderly population referred to a geriatric hospital. We divided the subjects into five groups based on their general functional status level as determined by performance-based repeated chair standing (RCS) test. According to standard test protocol, we performed the test with a standard chair (seat height 0.45 m). We asked the subjects to stand and sit five times consecutively "as fast as possible" with their upper extremities folded on their chest and recorded the time needed by the subjects to perform the task. We then assigned each subject into one of the groups, according to either the time each individual needed to complete the task or the subject's inability to perform it. Each subject then performed a series of single sit-to-stand (SSTS) trials in which we used the seat height to control the task difficulty. We controlled the strategy-related determinant by observing the use of the upper limbs and used three different strategies (no use of the arms, swinging of the arms, and pushing with the hands) and the inability to stand to describe the subject's performance.

Subjects within the two highest functional groups could complete the SSTS task at all seat heights with a slightly increased use of the swinging arms upper extremity strategy at the lowest seat height. When the difficulty of the task increased, the more functionally limited subjects were more affected and were forced to adopt compensatory movement strategies to perform the task successfully. The subject's functional status and task difficulty strongly influenced the effectiveness of the compensatory strategies. As seat height decreased, the compensatory movement strategies were less effective at maintaining sit-to-stand ability across the three lowest functional groups.

Results of our study clearly show the existence of a hierarchy of compensatory movement strategies in which the effectiveness of each strategy to maintain standing ability is strongly influenced by both the individual's general functional status and the difficulty of the sit-to-stand task. Some methodological considerations surfaced from our study. Subjects with an intermediate functional level and with shorter legs could complete the RCS test but were not able to stand when the seat was lowered at their shank heights. The results indicate that, to evaluate the relative status of all subjects properly when using instrumental performance-based tests, the seat should be height-adjustable and no single height should be fixed *a priori.*

Sit-to-stand and balance control motor strategies investigated in elderly stroke subjects
Mazzà, Lohmann Siegel, Brindle, Stanhope; in collaboration with Cappozzo
For the execution of a certain motor task, an individual selects a strategy among those that are consistent with the structural and functional constraints of his/her locomotor system and that tends to maximize the effectiveness of the motor act. The identification of the strategy allows for the assessment of the individual's functional status. Our study of a sample of hemiparetic stroke patients aims to identify the motor strategies adopted for execution of a sit-to-stand motor task followed by maintenance of the upright posture.

Asymmetric dynamic posture and movement is the most prevalent locomotor deficit of stroke-related hemiparesis. Weight bearing during dynamic tasks, such as rising from a chair, is usually compromised after a stroke. To understand the compensatory maneuvers that a person may use following a stroke, it is also important to understand the complex biomechanics underlying the performance of sit-to-stand transfer. To continuously measure weight-bearing ability and whole-body postural stability with minimum inconvenience for the patients, we decided to use only a force plate as a measuring instrument. We have already applied a similar approach to the problem of functional evaluation for the description of motor strategies used by young and elderly people to rise from a chair. Beginning by recording external forces only and using a musculoskeletal system model that is based on a telescopic inverted-pendulum (TIP) moved by a linear and two rotational muscle-equivalent actuators, we extracted parameters describing the kinematics and dynamics of the actuators. We identified different motor strategies in the two age groups and associated with both a different initial posture (ankle dorsiflexion angle) and speed of execution of the motor task. The proposed motor act, involving both dynamic and static balance control and the use of the TIP model to interpret the ground reactions data, shows potential to identify and evaluate movement alterations and the consequent functional limitations and level of disability in stroke patients.

Our study involves hemiplegic stroke patients, 55 to 65 years old, who suffered a stroke more than a year ago and who are medically stable and do not present movement disorders attributable to other neuropathies. Subjects are able to ambulate, at least at home, without aids, to rise from a chair with folded arms, and to stand for 60 seconds. Each study begins with the evaluation of strength, range of motion, and alignment of the joints. We then administer the NIH Stroke Scale questionnaire followed by a battery of performance tests (standing balance, repeated chair standing, walking speed) to assess the subject's functional ability level. According to the test results, we divide the subjects into three functional level groups. We performed the sit-to-stand test protocol after the screening. We collected anthropometric measurements (mass, height, thigh, shank, and foot length) for each subject. We then asked the subject to sit over a height-adjustable seat set at knee height and to choose the most comfortable foot position. We recorded knee and ankle angles and foot positioning and kept them constant for each subject among different trials. We next asked the subjects to rise from the chair while keeping their feet on the ground and their arms folded onto their chest and to perform the sit-to-stand motor task five times at a self-selected natural speed and five times at a self-selected high speed. Once the subjects were standing, we asked them to remain in the upright position for 60 seconds, facing a visual target.

We will feed the recorded ground reaction forces and anthropometric measures to the model and use its outputs to describe the motor strategies used by the subjects both before (in terms of trunk flexions and relevant velocity and momentum) and after (in terms of rotations and elevation of the whole body and both global muscular effort and coordination effort associated with the achievement of balance and raising) the "seat-off." We will then analyze the displacement of the center of pressure with respect to the affected side and make statistical comparisons between the different functional groups and investigate correlations with clinical evaluations.

Ultrasound examination of swallowing, oropharyngeal, and laryngeal activity in the fetus

Sonies, Miller; in collaboration with Macedonia

At birth, the normal-term neonate is able to suck and swallow for nutritional intake. The integrity of feeding skills is dependent on the prenatal development of orofacial, pharyngeal, and laryngeal anatomy that supports the emergence of respiratory and gastrointestinal tract functions that serve both the fetus and neonate. It is known that fetal swallowing contributes to homeostatic regulation of amniotic fluid volume, the acquisition and recirculation of intrauterine solutes, and overall fetal growth, which are influenced by a variety of maternal-fetal conditions that may also alter intrauterine aerodigestive function and potentially affect behaviors needed for postnatal feeding, swallowing, and respiration. Research has not yet investigated how these functions develop and when they become integrated. With advances in real-time noninvasive ultrasound imaging, we can examine these activities in the living human fetus.

In a collaboration with the National Naval Medical Center Prenatal Assessment Center aimed at investigating prenatal development of the upper aerodigestive anatomy and the association of emerging functions as predictors of postnatal feeding skills, we studied postnatal feeding and swallowing dysfunction in 62 randomly selected mothers with healthy fetuses 15 to 38 weeks gestational age and seven at-risk cases. We performed a standard clinical examination, collected biophysical measurements of fetal development , and performed a sonogram of upper aerodigestive structures observed during suckling, swallowing, and fetal breathing. All movements of the lips, jaw, tongue, larynx, and pharynx were video-recorded and observed for rate and periodicity. Four sonographic scan planes were used as well as power Doppler imaging to examine pulsations of the vascular anatomy and fluid flow

We distinguished fetal breathing patterns (nasal and oral) from swallowing and visualized respiration patterns of amniotic fluid flow through the nares and/or oral cavity. We developed behavioral classification criteria for observations of fetal behaviors, including tongue cupping, licking, suckling, mouthing, glottic flutter, swallowing, cough, and snort. We found significant linear regressions in pharyngeal and lingual growth across gestation while ingestive behaviors such as suckling emerged in a sequence of basic to complex movement patterns. Our data suggest that several ingestive behaviors emerge in a succession of basic to complex movement patterns and that the progression forms the basis for postnatal suckling and swallowing skills. For example, jaw and lip movements appeared first as simple opening and later progressed to repetitive open-close movements similar to excursion patterns found in infant suckling. Lingual movements increased in complexity from simple forward thrusting and cupping to anterior-posterior motions observed in suckling at term. Laryngeal movements varied from shallow flutter focused at the lumen to more complete abduction-adduction of the entire region. Our observations substantiate the premise that suckling, swallowing, and prephonatory movements are developing in preparation for extra-uterine survival. In the abnormal cases, we demonstrated delays in pharyngeal growth and a lack of pharyngeal movement during fetal swallowing and postnatal feeding complications. We postulate that prenatal developmental indices of emerging aerodigestive skills may guide postnatal decisions for feeding readiness and, ultimately, advance the care of the premature medically fragile neonate.

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PHYSICAL PRINCIPLES OF BIOMOLECULAR RECOGNITION, SELF-ASSEMBLY, AND REGULATION

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We study recognition reactions between biological macromolecules, with particular emphasis on the pathology of collagen fiber formation in brittle bone disease (osteogenesis imperfecta or OI) and other disorders. Over the years, we found that collagen interactions and fiber formation share a number of physical principles with interactions involving DNA and other biological helices. For instance, we discovered that the helical symmetry of the molecular backbone determines not only the pattern of spots on X-ray pictures but also sequence homology recognition, condensation and mesomorphism of DNA, and temperature-favored assembly and structure and stability of collagen fibers. Our main goal is to achieve a better understanding of these fundamental principles and to use the knowledge for diagnosis, characterization, and treatment of OI and other diseases. In collaboration with NICHD and extramural clinicians and scientists, we strive to bring our expertise in physical biochemistry and theory to molecular and cell biology and to clinical research and practice.

Thermal stability, metabolism, function, and pathology of type I collagen
Kuznetsova, Makareeva, Mertz, Leikin; in collaboration with Ananthanarayanan
Type I collagen is the most abundant animal protein; it forms the matrix of bone, skin, and other tissues. It was taken for granted that the type I collagen triple helix melts several degrees above body temperature in all species from Arctic fish to humans. However, we previously demonstrated that monomeric collagen slowly melts several degrees *below* body temperature (Leikina *et al.*, 2002). Thus, the collagen helix is even less stable than previously thought; its equilibrium state at body temperature is a random coil rather than a triple helix. Once its procollagen precursor is secreted from cells and processed by enzymes, a collagen helix begins to unfold. Micro-unfolding of the least stable domains appears to trigger self-assembly of fibers where helices are protected from complete unfolding. We showed that some collagen mutations cause substantial changes in micro-unfolding, resulting in abnormal fibrillogenesis (Kouznetsova, McBride, and Leikin, 2003).

More recently, we found that procollagen monomers are also thermally unstable at body temperature. The temperature and kinetics of procollagen and collagen denaturation appear to be similar. Therefore, procollagen cannot fold by itself at body temperature while cells have to use molecular chaperones to fold procollagen within the endoplasmic reticulum (ER). Almost nothing is known, however, about the mechanism of this process. The literature has reported several different chaperones that bind to collagen in the ER, but not whether any of them is responsible for the thermal stabilization of triple helix that allows collagen to fold. Among these collagen-binding ER chaperones, the only one believed to be collagen-specific is Hsp47. We are therefore currently focusing on investigating the thermal stability and folding of procollagen in the presence of Hsp47.

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Thermal stability of type I collagen from OI patients with glycine substitutions and deletions/insertions in the triple helix

Kuznetsova, Makareeva, Leikin; in collaboration with Cabral, Marini

The most common causes of moderate to lethal OI are glycine substitutions and deletions or insertions of small fragments in the $(Gly\text{-}X\text{-}Y)_n$ sequence of collagen triple helix. Many researchers reported changes in the thermal stability of collagen accompanying these mutations. However, thermal stability of mutant collagens was previously studied exclusively by the chymotrypsin/trypsin digestion assay. By comparing a variety of techniques and undertaking careful analysis of denaturation kinetics, we demonstrated that, while this assay yields useful information on rapid collagen micro-unfolding, it cannot be used for reliable evaluation of changes in the overall thermal stability of the protein. We therefore used differential scanning calorimetry and isothermal circular dichroism to continue our systematic analysis of the thermal stability of mutant collagens from the NIH collection of OI patients. We found that, depending on its location, the same substitution may reduce the melting temperature of collagen triple helix by 5°C or have virtually no effect on the melting temperature. Thus, it appears that the position of the mutation within certain domains is more important than the identity of the substituting residue or its immediate local environment. Interestingly, despite the fact that OI is caused primarily by mutations affecting the integrity of collagen triple helix, so far we have found no clear correlations between changes in the thermal stability of collagen helix and OI severity.

Abnormal matrix incorporation of type I collagen with triplet duplication mutation in lethal OI

Kuznetsova, Makareeva, Leikin; in collaboration with Cabral, Marini

A rare type of collagen mutation consists of a duplication or deletion of one or two Gly-X-Y triplets. These mutations do not interrupt the triplet sequence, and yet they cause severe clinical phenotypes. We previously reported that a Gly-Ala-Hyp triplet duplication at AA 868-876 in a lethal OI case results in a shift of register between collagen chains propagating through all 870 amino acids from the N-terminal end of the molecule to the mutation point (Cabral *et al.*, 2003). We found that the register shift causes a decrease in the melting temperature of about 2°C for helices with one mutant chain and about 6°C for helices with two mutant chains. It also alters protein micro-unfolding, fibrillogenesis, and kinetics of cleavage by N-proteinase and mammalian collagenase. An interesting observation was the complete absence of molecules with two mutant chains in fibers formed *in vitro* and the substantial reduction in the content of mutant molecules in matrix deposited by fibroblasts in tissue culture. By carefully monitoring the structural integrity of collagen triple helix during *in vitro* fibrillogenesis, we have now demonstrated that the molecules with two mutant chains denature faster than they can be incorporated into fibers.

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Disruption of collagen N-terminal folding domain in OI/EDS patients

Makareeva, Leikin; in collaboration with Cabral, Colige, Marini

A distinct subset of osteogenesis imperfecta patients present with mutations in the first 90 N-terminal residues of the type I collagen helix. In addition to the skeletal fragility of OI, the patients have characteristics of Ehlers-Danlos syndrome (EDS), including severe laxity of large and small joints and early-onset scoliosis. In seven children with OI/EDS, we determined that such mutations cause misfolding of collagen N-terminus, abnormal N-propeptide processing, incorporation of pN-collagen into matrix, and decreased diameter of dermal fibrils. Interestingly, in sharp contrast to mutations beyond the first 90 amino acids whose procollagen and collagen had identical thermal stability, six of the seven mutations resulted in different thermal stability of collagen and procollagen. *In vitro* cleavage of the six mutants with N-proteinase processed between 25 and 85 percent of proalpha1(I) chains. The seventh mutant with Gly88-Glu substitution appeared to be exactly at the border of the OI/EDS region. Its cleavage with N-proteinase was slower than in the normal control, but the cleavage was complete and the mutant exhibited similar thermal stability of procollagen and collagen. All seven mutants had delayed pericelluar processing, and their pN-collagen was incorporated into matrix deposited by cultured fibroblasts. Examination of the dermal fibers from six of the seven patients by electron microscopy found their diameters to be significantly smaller than those of matched controls, as is seen in EDS type VII.

The study defined an N-terminal folding region of collagen helix in which mutations alter not only the triple helical structure but also the secondary structure of the N-proteinase cleavage site. The retention of N-propeptide in a substantial proportion of collagen chains limits fibril diameter. The abnormal fibrils may cause laxity of joints and paraspinal ligaments either directly by reducing resistance to shearing forces or indirectly by altering interactions between collagen and other matrix components in the overlap zone of the D-periods.

Physical biochemistry of G349C collagen

Kuznetsova, Leikin; in collaboration with Cabral, Forlino, Marini

The first knock-in murine model for OI, the *brtl* mouse, was recently described. A classical glycine substitution was introduced into one *col1a1* gene, resulting in alpha 1(I) G349C chains. The *brtl* phenotype I is transmitted in an autosomal dominant fashion and resembles type IV human OI with wide variation in severity, from moderate to lethal. We used collagen from these mice to continue our structural and functional studies of collagen and for comparison with collagen from *oim* mice (the only other existing nonlethal murine OI model). We found that *brtl* collagen molecules with two mutant chains and normal molecules were equally well incorporated into the matrix and formed mature covalent cross-links with the same efficiency. We detected only minimal posttranslational overmodification of *brtl* collagen in cell cultures and in animal tissues, which was similar in lethal and nonlethal animals. We found changes neither in thermal stability or the rate of thermal denaturation nor in any collagen-collagen recognition or interactions. We observed disruption in quasi-crystalline lateral packing of *brtl* tendons, but only in some, mostly prepubertal, mutant animals. In contrast, our studies of *oim* collagen by similar techniques (Kouznetsova, McBride, and Leikin, 2001 and 2003) revealed substantial changes in collagen stability, fibrillogenesis, and collagen-collagen interactions as well as more pronounced disruption of tendon crystallinity. Nevertheless, while G349C substitution leads from moderate to lethal OI in heterozygous animals, heterozygous *oim* mice are almost asymptomatic.

Overall, our results suggest that OI symptoms and phenotype variation in G349C animals are related to abnormal interactions of mutant collagen helices with other matrix molecules or abnormal function of osteoblasts rather than to abnormal structure, physical properties, or interactions between mutant collagen helices. We are presently conducting further studies of the pathology mechanism and phenotype variation in *brtl* mice. Our preliminary data indicate that collagen helices with one mutant chain might be abnormally retained inside cells and disrupt osteoblast function.

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Sequence homology recognition and sequence-specific effects in DNA condensation

Leikin; in collaboration with Cherstvy, Kornyshev

It was recently established that pairing of homologous fragments of intact, duplex DNA in cells precedes homologous recombination. It was argued that the pairing is caused by some direct DNA-DNA interaction. In an attempt to understand the possible nature of such an interaction, we previously showed that electrostatic forces between intact DNA duplexes are sequence-dependent and may lead to efficient sequence homology recognition between 50 to 200 base pair fragments (Kornyshev and Leikin, 2001). However, the original theoretical predictions for interaction between two isolated DNA fragments were difficult to verify experimentally. We therefore extended the theory to the formation of DNA aggregates. Our calculations showed that homologous fragments should exhibit aggregation at a slightly lower concentration of condensing counterions and that the structure of such aggregates should be distinguishable from the structure of aggregates formed by nonhomologous DNA fragments. Our predictions rationalized several previous experimental observations, e.g., torsional deformation of DNA in aggregates. Most important, we formulated several predictions for measurable parameters of DNA aggregation, which can be tested in straightforward *in vitro* experiments. These experiments are presently under way.

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Mesoscopic dielectric response in biomolecular systems

Mertz; in collaboration with Krishtallik

Numerous studies published in the literature and our own studies of collagen and DNA interactions showed that electrostatic forces often play an important role in the structure and function of biological macromolecules. However, proper application of electrostatic theory to understanding the corresponding phenomena is hindered by numerous difficulties inherent in the application of macroscopic physics at the microscopic and mesoscopic distance scales typical for biomolecular interactions. For instance, our previous experimental studies of dielectric response in structured polar liquids (such as water) to charge redistribution within a spectroscopic probe showed that existing dielectric theories are in qualitative disagreement with the data. We therefore developed a novel formulation for the description of dielectric response in such liquids, which appears to be consistent with our and other observations. Furthermore, by deriving continuum electrostatics models of proteins from first principles, we demonstrated the inadequacy of traditional approximations. Preliminary analysis suggests that our calculations, made without any adjustable parameters, are in satisfactory agreement with the existing data, e.g., for redox potentials of various cytochromes *c*.

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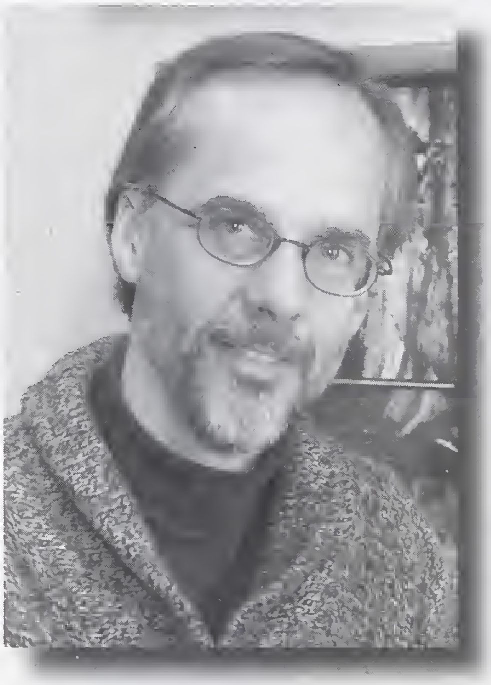
NERVOUS SYSTEM DEVELOPMENT AND PLASTICITY



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Our research is concerned with understanding the molecular mechanisms by which functional activity in the brain regulates development of the nervous system during late stages of fetal development and early postnatal life. The main objectives of our research program are (1) to understand how the expression of genes controlling the developing structure and function of the nervous system are regulated by patterned neural impulse activity and (2) to determine the functional consequences of neural impulse activity on major developmental processes, including cell proliferation, survival, differentiation, growth cone motility, axon bundling (fasciculation), neurite outgrowth, synaptogenesis, synapse remodeling, myelination, interactions with glia, and the mechanisms of learning and memory in postnatal animals.

Action potential regulation of glial development and myelination

Stevens, Dakin, Weinberg, Fields; in collaboration with Gallo

The importance of neural impulse activity in regulating development of neurons is widely appreciated, though not in the case of the development of non-neuronal cells (glia). Our recent work has examined the possible influence of axonal impulse activity on developing glia, which form myelin in the peripheral (PNS) and central nervous system (CNS). Myelin is the spiral wrapping of membrane around axons that provides electrical insulation essential for rapid impulse conduction. Curiously, published evidence suggests that action potentials may inhibit myelination in the PNS but stimulate myelination in the CNS; however, the results are controversial. The mechanism for this activity-dependent axon-glial communication is unknown, as are the molecular mechanisms that would mediate effects of action potentials on myelination. We investigated this apparent paradox by taking advantage of a preparation of DRG neurons that can be stimulated in cell cultures fitted with platinum electrodes. The central axons of DRG neurons are myelinated by oligodendrocytes, and their peripheral axons are myelinated by Schwann cells. Our findings confirm the opposite effects of impulse activity in myelination of CNS and PNS and show that different purinergic signaling molecules released by axons in an activity-dependent manner control differentiation of myelinating glia in the PNS and CNS.

Our studies on Schwann cells show that, in the PNS, extracellular ATP released from axon firing action potentials acts on purinergic receptors on Schwann cells (P2Y receptors), causing an increase in intracellular calcium, activation of transcription factors, and regulation of genes involved in Schwann cell differentiation. The result is that several developmental processes in Schwann cells are regulated by the release of ATP from electrically active axons, including inhibiting Schwann cell proliferation, arresting cellular differentiation at an immature stage, and inhibiting myelination.

Our studies on CNS myelination reveal that oligodendrocyte progenitor cells (OPCs) can detect impulse activity in premyelinated axons and respond with an increase in intracellular calcium. We identified the axon-glial signaling molecules involved and found that adenosine, a metabolite of ATP, is of primary importance. Using RT-PCR, we detected all four types of adenosine receptor in purified cultures of OPCs and in acutely dissociated OPCs from transgenic mice. Impulse activity inhibited cell proliferation of myelinating glia in both the PNS and CNS, but adenosine derived from electrically active axons, rather than ATP as in the PNS, was responsible for inhibiting proliferation of myelinating glia in the CNS. Moreover, the effects of impulse activity on differentiation and myelination were opposite in Schwann cells and OPCs. In contrast to Schwann cells, in which our experiments showed that electrical activity arrests differentiation, we found that action potentials acting through adenosine receptors promoted differentiation of OPCs to the mature stage. When cultures of DRG neurons were myelinated by oligodendrocytes, we found that the addition of adenosine significantly increased myelination. Even a transient exposure of OPCs to adenosine stimulates differentiation and increases myelination by oligodendrocytes.

Our results showing that different extracellular signaling molecules mediate axon communication with OPCs in the CNS and with Schwann cells in the PNS helps resolve a long-standing controversy on the opposite effects of impulse activity on myelination in the brain and PNS. Our research identifies adenosine as a potent axon-derived differentiation factor for OPCs that operates in an activity-dependent manner and promotes myelination. Together with our research detecting several types of purinergic receptors in OPCs, these findings open new avenues of research into oligodendrocyte development with potential for new therapeutic approaches to treating demyelinating disease.

Fields RD, Stevens-Graham B. New insight into neuron-glia communication. *Science* 2002;298:556-562.

Stevens B, Porta S, Haak LL, Gallo V, Fields RD. Adenosine: a neuron-glial transmitter promoting myelination in the CNS in response to action potentials. *Neuron* 2002;36:855-868.

Intracellular signaling in conversion of short-term memory into long-term memory

Dudek, Fields

It is widely appreciated that there are two types of memory: short term and long term. It has been well known for decades that gene expression is necessary for converting short-term into long-term memory, but it is not known how signals reach the nucleus to initiate the conversion process. Current theory holds that, in response to firing a synapse in the appropriate manner to generate a short-term memory, a signaling molecule is generated from the submembrane region of the synapse, which travels up the dendritic tree to the cell body. After entering the nucleus, the signaling molecule is presumed to initiate gene transcription by activating the transcription factor CREB. The gene product is synthesized and distributed throughout the dendritic tree, but the protein can recognize synapses that have been temporarily strengthened, rendering the change permanent.

We proposed an alternative hypothesis based on our studies of action potential–dependent intracellular signaling and CREB activation in DRG neurons. The hypothesis proposed that, rather than generating a specialized synapse-to-nucleus signaling molecule, *somatic* action potentials could be responsible for activating gene transcription to convert short-term into long-term memory via the same intracellular signaling pathway that we previously studied in DRG neurons. We tested the hypothesis by backfiring axons from CA1 hippocampal neurons in slice preparations in the absence of glutamate neurotransmitter blockers, causing neurons to fire somatic action potentials in the absence of all excitatory synaptic input in brain slice preparations. Following stimulation, we analyzed the slices by immunocyochemistry for activation of CREB and other signaling enzymes involved in long-term potentiation (LTP) of synapses as well as for expression of genes closely associated with LTP induction. The results showed that backfiring the axons by antidromic stimulation was sufficient to activate CREB and the signaling enzyme MAPK in CA1 neurons in the absence of all excitatory synaptic stimulation. When slices were stimulated antidromically, the outcome was synaptic stimulation that normally results in a transient strengthening of the synapse (e-LTP) rather than a long-lasting strengthening of the synapse (l-LTP). These results, which support our hypothesis and eliminate the necessity of a synapse-to-nucleus signaling molecule for l-LTP, are consistent with the original theory of Hebb that states that the firing of a neuron determines whether or not it forms a stronger synaptic connection with another neuron.

Dudek MS, Fields RD. Mitogen-activated protein kinase/extracellular signal-regulated kinase activation in somatodendritic compartments: roles of action potentials, frequency and mode of calcium entry. *J Neuroscience* 2001;21.RC122:1-5.

Dudek S, Fields RD. Somatic action potentials are sufficient for late-phase LTP-related cell signaling. *Proc Natl Acad Sci USA* 2002;99:3962-3967.

Gene expression in hippocampal synaptic plasticity

Cohen, Lee, Fields; in collaboration with Becker

Long-term potentiation (LTP) and long-term depression (LTD) are two widely studied forms of synaptic plasticity that can be recorded electrophysiologically in the hippocampus; these phenomena are believed to represent a cellular basis for memory. We are using custom-made cDNA microarrays to investigate the signaling pathways, genes, and proteins involved in these forms of synaptic plasticity. Our research has identified sets of transcription factors, structural genes, and signaling pathways that are regulated specifically by activity patterns leading selectively to different types of synaptic plasticity. The objective of our research is to understand the how gene-regulatory networks are controlled by the appropriate patterns of impulses leading to different forms of synaptic plasticity and to identify new molecular mechanisms regulating synaptic strength.

Cohen JE, Fields RD. Extracellular calcium depletion in synaptic transmission. *The Neuroscientist* 2003; in press.

Regulation of gene expression by action potential firing patterns

Lee, Tendi, Cohen, Fields; in collaboration with Becker, Waxman

Using custom cDNA arrays for gene expression profiling, we have investigated how gene expression is regulated by patterned action potential firing in neuronal cell culture. The pattern of impulse activity is regulated by electrical stimulation through platinum electrodes in specially designed cell culture dishes. After stimulation, we measure mRNA and protein expression by gene arrays, quantitative RT-PCR, Western blot, and immunocytochemistry. Our work tests the hypothesis that gene transcription can be regulated by the pattern of action potential firing. The results obtained thus far reveal signaling pathways and gene regulatory networks that respond selectively to appropriate temporal patterns of action potential firing in neurons. Our findings provide a better understanding of how nervous system development and plasticity may be regulated by information coded in the temporal pattern of impulse firing in the brain.

Eshete F, Fields RD. Spike frequency decoding by autonomous activation of CaMKII in DRG neurons. *J Neurosci* 2001;21:6694-6705.

Klein JP, Tendi EA, Black JA, Fields RD, Waxman SG. Patterned electrical activity modulates sodium channel expression in sensory neurons. *J. Neurophysiology* 2003;74:192-198.

Neurofibromatosis

Lee, Tendi, Cohen; in collaboration with Becker, DeVries

Neurofibromatosis (NF-1) is an autosomal dominant condition leading to nerve sheath tumors that can develop into malignant cancer. We are using gene expression profiling in combination with quantitative RT-PCR and biochemical methods to investigate the processes involved in the transition from a benign to a malignant tumor. We are using three types of custom-made microarrays of genes involved in cell signaling, immune system function, and nervous system expression to compare mRNA expression in normal human Schwann cells and a Schwann cell line derived originally from an NF-1 patient who died from a highly malignant form of neurofibromatosis. Our studies identify hundreds of genes that are dysregulated in the malignant Schwann cells, including those associated with all aspects of cellular function associated with malignancy, such as, for example, those regulating cell proliferation, motility, growth factor, and immune system responses.

Stevens B, Fields RD. Regulation of the cell cycle in normal and pathological glia. *The Neuroscientist* 2002;8: 93-97.

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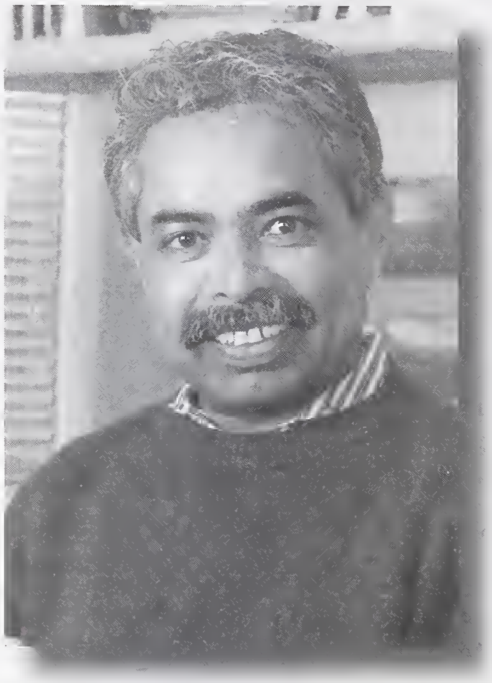
CALCIUM-BASED EXCITABILITY IN GLIAL CELLS



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We are interested in studying the cellular mechanisms of calcium signaling by glial cells in the nervous system. Glial cells and neurons are in intimate communication with each other during central nervous system (CNS) development and normal brain function. Glial cells monitor and respond to neural activity by conditioning the extracellular milieu, signaling within glial cell networks, and sending signals back to neurons. Such signaling is in the form of propagated Ca^{2+} waves that spread over long distances in response to synaptic activity. One of our objectives is to understand the processes that support temporal and spatial characteristics of Ca^{2+} signals within cells and between cells. A second objective is to understand the nature of glial cell signals in response to neuronal activity and the consequence of such signals to CNS function.

Ca^{2+} signaling microdomains

We have previously shown that Ca^{2+} wave propagation occurs through a series of Ca^{2+} signaling microdomains organized along glial cell processes. The microdomains act as highly specialized Ca^{2+} release sites that produce discretely localized spurts of cytoplasmic Ca^{2+} increases. In this way, wave propagation occurs by a fire-diffuse-fire cascade whereby the signal leaps along in a salutatory fashion. The sites are found five to seven micrometers apart and are characterized by high-density patches of endoplasmic reticulum (ER) proteins involved in Ca^{2+} signaling, such as the inositol 1,4,5-trisphosphate receptors (IP_3Rs), sarco-endoplasmic reticulum calcium pumps, calreticulin, and at least one mitochondrion in close association. In addition to supporting long-distance wave propagation, the specialized sites provide for locally discrete Ca^{2+} signals, which last for only very brief periods. The proteins that make up the signaling microdomain and the mechanisms by which they support the highly organized Ca^{2+} release are not yet understood.

Haak LL, Grimaldi M, Smaili SS, Russell JT. Mitochondria regulate Ca^{2+} wave initiation and inositol trisphosphate signal transduction in oligodendrocyte progenitors. *J Neurochem* 2002;80:405-415.

Holtzclaw LA, Pandhit S, Bare DJ, Mignery GA, Russell JT. Astrocytes in adult rat brain express type 2 inositol 1,4,5-trisphosphate receptors. *Glia* 2002;39:69-84.

Molecular characterization of signaling rafts

Verbois, Weerth, Holtzclaw*

Our current focus is to investigate the molecular organization of Ca^{2+} signaling microdomains and to describe their functional regulation in detail. The overall aim of this investigation is to test the hypothesis that, in the specialized microdomains of Ca^{2+} release, a number of macromolecular protein complexes are physically brought together, in different membrane systems and interact to generate large spatially restricted Ca^{2+} signals. We have focused on glial cell processes, particularly cells of the oligodendrocyte lineage and astrocytes. We propose to undertake a molecular characterization of these specialized Ca^{2+} release sites to determine the interacting protein assemblies that orchestrate this process. In addition, we propose to probe the functional significance of unique proteins found in the macromolecular assembly. We will investigate if the microdomains are preferentially localized to predestined cellular sites of contact to support optimal signaling between neurons and glial cells and between glial cells.

To isolate oligomeric protein assemblies that make up specialized microdomains of Ca^{2+} signaling in intact form from glial cell membranes isolated from mouse brains, we used three separate strategies to obtain glial cell membranes. In one, we obtained a total membrane preparation from isolated rat cortices by differential centrifugation to remove unbroken cells, connective tissue, nuclei, and mitochondria. We used the pellet containing membranes as starting material to solubilize protein complexes by using different detergent solutions. In a second method, we prepared cell-specific membranes from brain homogenates by using an affinity method; we used antibodies against cell surface proteins, namely, Ran-2, an astrocytic surface antigen, or NG-2, an OP-cell antigen, for specifically immunopanning the homogenate to obtain subfractions containing membranes enriched in astrocytic membranes or OP-cell membranes. To obtain isolated membranes, we used purified cultures containing either astrocytes or OP cells, which were solubilized in 1 percent Triton X-100 at a detergent-to-protein ratio of 1:2 (w/w) in ice for one hour. The solubilized material was applied on an Optiprep density gradient and centrifuged to separate raft-like membrane domains.

We fractionated the gradients and tested each fraction in Western blots for protein content. We used caveolin-1 as a marker for raft-like membranes and, using specific antibodies, probed for a number of proteins involved in Ca^{2+} signaling, including the surface membrane receptors M1 and P2Y1, the surface membrane ion channel TrpC1, the heterotrimeric G-protein isoform Gq, scaffolding protein, homer, and the endoplasmic reticulum membrane proteins IP3R2 and RyR3. The analysis showed that most of the proteins involved in Ca^{2+} signaling separated into low-density regions of the gradient, which also contained caveolin-1 (see Figure 25.1). The data indicate that these signaling proteins might exist in oligomeric complexes with each other in lipid

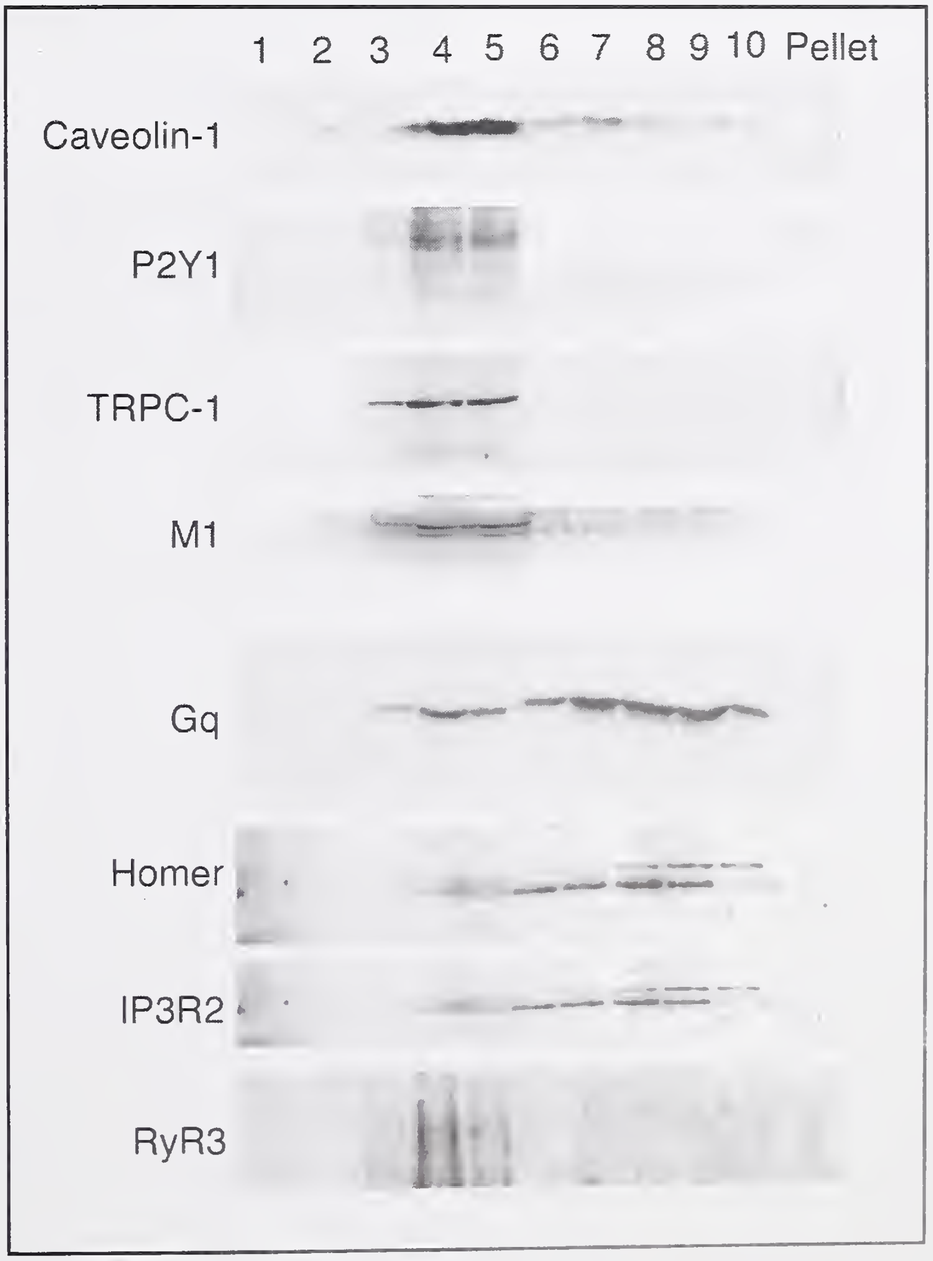

FIGURE 25.1

Proteins involved in CA^{2+} signaling, identified by Western blot.

domains that are cholesterol-rich and raft-like. We are investigating the possibility that these proteins occur in caveolin-rich lipid rafts.

We plan to use the gradient fractions as starting material and to immunoprecipitate by using specific antibodies against IP3R2, M1, and TrpC1 to determine the components that occur in oligomeric complexes. The experiment will also test if caveolin interacts with Ca^{2+} signaling proteins. Specifically, we will again analyze immunoprecipitated proteins in Western blots followed by high-resolution molecular characterization using proteomic technologies.

The technology for proteomics involves separation of protein components in the macromolecular assembly, followed by molecular identification of components using spectrometry. We will separate proteins by 1D PAGE, identify protein bands by staining and excise them, and then digest them with trypsin. We will identify peptide pools in the tryptic digests by mass spectrometry using MALDI-TOF and will compare peptide mass data with protein databases for positive hits. Tandem LC-MS/MS is another technique that will allow for peptide mass determination and database comparison. After molecular identification, we plan to select interesting subsets of proteins to investigate their cellular localization and functional importance. We now focus on myelianting glia in central and peripheral nerves as well as on astrocytic processes *in situ*. Initial experiments will involve immunocytochemical analysis of protein distribution in the context of the structural arrangement of glial cell membranes in the neuronal circuits and myelinated nerves.

Wang C-Y, Yang F, He X, Chow A, Du J, Russell JT, Lu B. Ca^{2+} binding protein frequenin mediates GDNF-induced potentiation of Ca^{2+} channels and transmitter release. *Neuron* 2001;32:99-112.

Ca^{2+} signaling between axons and myelinating glia

Toews

We have recently begun to characterize signaling between axons and their myelinating glia (Schwann cells and oligodendrocytes) in the peripheral and central nervous systems. We have focused on Ca^{2+} signaling and started to study the axoglial apparatus in the nodes of Ranvier. We have selected antibodies against proteins involved in Ca^{2+} signaling (e.g., IP3Rs and RyRs) and known structural proteins unique to different regions of the node and are investigating the discrete localization of the proteins by using immunocytochemistry followed by high-resolution fluorescence microscopy.

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INDEX OF PRINCIPAL INVESTIGATORS

Greti Aguilera 31
Tamas Balla 58
Jeffrey Baron 35
Peter Basser 217
Sergey Bezrukov 300
Carolyn Bondy 39
Juan Bonifacino 6
Robert Bonner 224
Marc Bornstein 142
Andres Buonanno 165
Michael Cashel 247
Kevin Catt 61
Hao-Chia Chen 66
Leonid Chernomordik 101
Ajay Chitnis 250
Janice Chou 86
Andreas Chrambach 104
George Chrousos 309
David Clark 282
Robert Crouch 254
Mary Dasso 183
Igor Dawid 257
Melvin DePamphilis 286
Thomas Dever 186
Maria Dufau 68
Douglas Fields 346
Amir Gandjbakhche 228
Ramanujan Hegde 10
Alan Hinnebusch 190
Dax Hoffman 122
Bruce Howard 290
Kuo-Ping Huang 72
Catherine Jackson 14
Stephen Kaler 139
Rohinton Kamakaka 196
Judith Kassis 260
James Kennison 263
David Klein 168
Michael Lamb 147
Chi-Hon Lee 198
Sergey Leikin 342
Henry Levin 202
Judith Levin 266
Mary Lilly 17
Jennifer Lippincott-Schwartz 21
Peng Loh 174
Paul Love 237
Bai Lu 124
Richard Maraia 293
Leonid Margolis 107
Joan Marini 3
Mark Mayer 119
Chris McBain 128
Anil Mukherjee 89
Elaine Neale 178
Lawrence Nelson 44
Phillip Nelson 180
John Newman 151
Lynnette Nieman 316
Ralph Nossal 234
Ida Owens 94
Keiko Ozato 296
Karel Pacak 320
Adrian Parsegian 303
Karl Pfeifer 241
Forbes Porter 97
Donald Rau 306
Owen Rennert 133
John Robbins 158
Roberto Romero 327
Tracey Rouault 25
James Russell 350
Thomas Sargent 270
Rachel Schneerson 160
James Segars 325
Yun-Bo Shi 206
Steven Stanhope 332
Stanko Stojilkovic 76
Mark Stopfer 132
Gisela Storz 28
Constantine Stratakis 48
Charles Strott 81
Alexander Strunnikov 209
Stephen Suomi 154
Brant Weinstein 273
Robert Weisberg 278
Heiner Westphal 244
Roger Woodgate 211
Jack Yanovski 54
Alfred Yergey 111
Joshua Zimmerberg 115

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